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(54) Title: LYSOPHOSPHATIDIC ACID ACYLTRANSFERASE GENE AND ITS USE		
(57) Abstract An assay for an inhibitor or activator of inflammation mediated via lysophosphatidic acid acyltransferase (LPAAT) which utilises recombinant human LPAAT. The recombinant human LPAAT is brought into contact with a candidate inhibitor or activator in the presence of a lysophosphatidic acid substrate and a fatty acid cofactor and the amount of LPAAT activity in the presence and absence of the inhibitor or activator is compared. Isolated LPAAT polypeptides, polynucleotides encoding LPAAT and vectors from which the LPAAT is expressed are provided.		

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LYSOPHOSPHATIDIC ACID ACYLTRANSFERASE GENE AND ITS USE5 FIELD OF THE INVENTION

The invention relates to the human lysophosphatidic acid acyltransferase gene, the protein encoded by the gene and an assay using the lysophosphatidic acid
10 acyltransferase protein.

BACKGROUND TO THE INVENTION

Endotoxic lipopolysaccharides and other cell wall
15 components of pathogenic organisms induce a cascade of endogenous inflammatory mediators that lead to a condition defined as the systemic inflammatory response syndrome (SIRS) which is associated with multiple organ dysfunction [see references in (Rice et al., 1994b)]. A number of
20 studies involving direct infusion or, conversely, the use of specific inhibitors in animal models have suggested that the primary endogenous mediators of SIRS are tumour necrosis factor α (TNF α) and interleukin 1 β (IL-1 β). These primary endogenous mediators are synergistic and para- and
25 autostimulatory. Endotoxin and other bacterial products themselves are direct cellular activators and may mimic many of the actions of TNF α and IL-1 β . Most therapeutic interventions developed for sepsis and SIRS have targeted only single components of the monokine cascade and in
30 preclinical models require administration either prior to or within 1-2 h following endotoxin for significant protection [see references in (Rice et al., 1994b)].

An anti-inflammatory approach is to suppress intracellular signal transduction pathways used by several
35 inflammatory mediators involved in SIRS. Recent studies

have shown that IL-1 β (Bursten et al., 1991), TNF α (Bursten et al., 1994), platelet activating factor (Tou et al., 1991), as well as bacterial cell wall products such as lipid A or LPS (Bursten et al., 1992) may activate and
5 signal, at least in part, through a common lipid intracellular signalling pathway. Activation of this pathway occurs within seconds of exposure of cells to a stimulus and leads to rapid increases in intracellular levels of specific species of phosphatidic acid (PA) and
10 diacylglycerol (DAG).

While a major function of phospholipids is to form biological membranes, a subclass of phospholipids and their metabolites have been implicated as signalling molecules, acting either as intracellular second messengers or as
15 extracellular agonists that modulate cell function (Jalink et al., 1994; Divecha and Irvine, 1995; Moolenaar, 1995; English et al., 1996). LysoPA (LPA) or 1-acyl-*sn*-glycerol-3-phosphate consists of a glycerol backbone with a fatty acyl chain at the *sn*-1 position, a hydroxyl group at the
20 *sn*-2 position and a phosphate group at the *sn*-3 position (Fig.1). In the endoplasmic reticulum (ER) membrane, LPA is formed from glycerol-3-phosphate (G3P) through the action of glycerol-3-phosphate acyltransferase (GPAT). LPA is then further acylated in the ER by lysophosphatidic acid
25 acyltransferase (LPAAT), also named 1-acyl-*sn*-glycerol-3-phosphate-acyltransferase, to yield PA, the precursor of all glycerolipids. The rate of acylation of LPA to PA is very high and, consequently, there is little accumulation of LPA at the site of biosynthesis. PA can either be
30 hydrolysed by phosphatidic acid phosphohydrolase (PAP) to yield DAG or, alternatively, can be converted to CDP-DAG for the synthesis of more complex phospholipids in the ER (Fig.1), from which they are transported to different subcellular compartments [for review see (Jalink et al.,

1994)] .

LPA is the simplest of all glycerophospholipids. While LPA has long been known as a precursor of phospholipid biosynthesis in both eukaryotic and
5 prokaryotic cells, only recently has LPA emerged as an intracellular signalling molecule that is rapidly produced and released by activated cells, notably platelets, to influence target cells by acting on a specific cell-surface receptor (Jalink et al., 1994; Moolenaar, 1995) .

10 Although its precise physiological (and pathological) functions *in vivo* remain to be explored, LPA derived from platelets has all the hallmarks of an important mediator of wound healing and tissue regeneration. In addition to acting as an autocrine stimulator of platelet aggregation,
15 LPA stimulates the growth of fibroblasts, vascular smooth muscle cells, endothelial cells, and keratinocytes; furthermore, it promotes cellular tension and cell-surface fibronectin binding, which are important events in wound repair. As a product of the blood-clotting process, LPA is
20 a normal constituent of serum (but not platelet-poor plasma), where it is present in an albumin-bound form at physiologically relevant concentrations, and possibly accounts for much of the platelet-derived biological activity of serum [for review see (Jalink et al., 1994;
25 Moolenaar, 1995)] .

PA is an intracellular lipid closely related in structure to DAG. PA has been shown to be induced in a variety of cell types in response to a diverse group of activating factors, including LPS, $\text{TNF}\alpha$, $\text{IL-1}\beta$, platelet
30 derived growth factor, IL-8, hypoxia/reoxygenation, and platelet activating factor (Singer et al., 1994). PA species are potent growth factor mimetic molecules in some systems, and have been shown to inhibit *ras-gap* and *rho-gap* interactions, induce enhanced expression of *fos* and *myc*
35 [see references in (Bursten et al., 1994)], mobilise Ca^{2+}

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flux, and activate NADPH oxidase, phospholipase C (PLC), protein kinase C and phosphatidylinositol-4-phosphate kinase.

PA also induces a number of cellular functions, including hormone release, actin polymerisation, platelet aggregation, muscle contraction and gene transcription [for review see (English et al., 1996)]. PA species, when added exogenously, can act as potent mitogens, alter cellular phenotype, or change cytoskeletal dynamics [see references in (Bursten et al., 1994)]. These PA species consist of heterogeneous sub-classes and vary extensively in bond type (i. e., *sn*-1 ether vs. vinyl ether vs. ester), acyl chain length, and the degree of saturation of the acyl chains in their substituted *sn*-1 and *sn*-2 positions [for references see (Bursten et al., 1994)]. PA can be produced by this *de novo* synthesis or alternatively, by phospholipase D (PLD) hydrolysis of phospholipids, or by the action of DAG kinase on DAG [for review see (Jalink et al., 1994; Divecha and Irvine, 1995)].

The heterogeneity of PA structures is in part due to this relatively large number of sources and means for cellular production of PA. For instance, PA species containing linoleate or oleate in the *sn*-2 position appear to be derived from LPAAT, which is induced by LPS (Bursten et al., 1992) or IL-1 (Bursten et al., 1991) in rat or human mesangial cells. In P338 monocytic leukaemia cells, IL-1 β stimulated PA species (after 15 seconds) are mainly composed of C18 at the *sn*-1 position and predominantly linoleoyl at the *sn*-2 position. However, in these cells TNF α stimulation gives a similar, but somewhat more complex response in terms of PA species noted at 15 seconds, with a greater contribution of oleate and alkyl PA species to the PA species mix (Bursten et al., 1994).

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Farese et al. (1987) have described that insulin increases *de novo* PA synthesis within 5 to 10 seconds; within 1 minute, this synthesis is 60 times greater than that of controls. Thus, they propose that the initial
5 increase in DAG is due to both increased hydrolysis of phospholipids and a burst of *de novo* PA synthesis and after 5 to 10 minutes, *de novo* PA synthesis continues as a major source of DAG. They conclude that both phospholipid effects of insulin seem important for generating DAG and other
10 phospholipid-derived intracellular signalling substances.

Recently, LPAAT inhibitors, such as lisofylline and pentoxifylline, have been shown to block the inflammatory response produced by an increase in PA. Pentoxifylline (PTX) is a weak inhibitor of PA induction either by IL-1 β
15 or LPS stimulation (Rice et al., 1994a; Rice et al., 1994b). PTX has been shown to confer protection in inflammatory conditions such as septic shock, respiratory distress syndrome, and experimental autoimmune encephalomyelitis. A novel PTX metabolite, CT-1501R (or
20 lisofylline), the R enantiomer of the first metabolite (M1) of PTX, was detected in patients treated with PTX in conjunction with ciprofloxacin. Lisofylline was not detectable in patients treated with PTX alone. Increasing levels of lisofylline in patients treated with PTX and
25 ciprofloxacin appeared to correlate with the ability to prevent multiorgan dysfunction in patients treated with IL-2 [see references in (Rice et al., 1994b)]. When tested in a mouse monocyte cell line P388 stimulated with LPS, lisofylline was 800-fold more potent at inhibiting PA and
30 DAG generation than was PTX (Rice et al., 1994a). Lisofylline inhibits the action of (Singer et al., 1994) and the production of (Rice et al., 1994b) inflammatory cytokines. Lisofylline inhibits organ damage from cancer therapy, accelerates haematopoietic recovery and prevents

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multiorgan dysfunction during acute inflammation (Singer et al., 1994). Lisofylline has been tested in a mouse model of endotoxin lethality and significantly improved survival was observed even when administered four hours following an LD₉₅ dose of endotoxin (Rice et al., 1994a). Lisofylline treatment prevents hypoxia-induced PA increases and LPA consumption in human neutrophils, and prevents neutrophil chemotaxis and adherence *in vitro*, and lung injury and lung neutrophil accumulation in mice subjected to haemorrhage and resuscitation. In addition, lisofylline treatment prevents increases in mRNA levels and protein production of a variety of proinflammatory cytokines in multiple lung cell populations after blood loss and resuscitation (Abraham et al., 1995). The later results indicate the fundamental role of PA metabolism in the development of acute inflammatory lung injury after blood loss.

Although LPAAT thus appears to be a target for blocking the inflammatory response this protein has not been isolated and the gene encoding the protein has not been identified. Thus present assays for screening putative inhibitors of LPAAT are based on the use of crude cell extracts containing the enzyme. Such assays are disclosed in, for example, WO95/13075, the contents of which are incorporated herein by reference.

25

SUMMARY OF THE INVENTION

G15 is one of a number of genes which has been mapped to a region of the human chromosome between the HLA class II region and the C4 genes of the human MHC complex (Campbell and Trowsdale, 1993). The sequence and function of this gene was previously unknown.

We have analysed the G15 gene and found that it encodes a protein which has homology to bacterial LPAAT (1-acyl-sn-glycerol-3-phosphate acyltransferase (also:

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lysophosphatidic acid acyl transferase)). We have confirmed that this novel protein has LPAAT activity. The present invention thus makes available for the first time a recombinant source of the human and other mammalian version of this enzyme.

The molecular cloning of a mammalian LPAAT thus allows the provision of improved assay systems for therapeutic compounds. The information provided by the present invention also allows other structural and functional information about the enzyme to be obtained and used as described herein.

Accordingly the present invention provides an assay for an inhibitor of inflammation mediated via LPAAT, which assay comprises the steps of:

(a) bringing human LPAAT expressed from a vector comprising a nucleic acid sequence which encodes said LPAAT and which is operably linked to a promoter heterologous to said sequence into contact with a candidate inhibitor in the presence of a lysophosphatidic acid (lysoPA) substrate and a fatty acid cofactor under conditions where, in the absence of inhibitor, the lysoPA is converted to phosphatidic acid (PA), and

(b) comparing the amount of LPAAT activity in the presence and absence of said candidate inhibitor.

The invention further provides an assay for an inhibitor of inflammation mediated via LPAAT, which assay comprises the steps of:

(a) bringing human LPAAT expressed from a vector comprising a nucleic acid sequence which encodes said LPAAT and which is operably linked to a promoter heterologous to said sequence into contact with a candidate activator in the presence of a lyso phosphatidic acid (lysoPA) substrate and a fatty acid cofactor under conditions where, in the absence of activator, the lysoPA is converted to phosphatidic acid (PA), and

(b) comparing the amount of LPAAT activity in the

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presence and absence of said candidate activator.

These assays can provide easy screening assays suitable as a high throughput assay for LPAAT inhibitors or activators. The LPAAT may preferably be present in
5 growing or intact cells in which the LPAAT has been expressed or in an extract or microsomal fraction from the said cells. In a preferred embodiment, the cells have little or no endogenous LPAAT activity for the substrate. The cells may have substantially no endogenous LPAAT
10 activity for the substrate. Such cells include insect cells. Alternatively, the assay may be conducted using mammalian cells, such as CHO, COS, P388 or HepG2 cells in which the LPAAT is expressed.

In a particularly preferred aspect of the invention
15 we have found that when LPAAT is expressed in COS cells, membrane vesicles containing recombinant LPAAT are produced. Thus the assay may be conducted wherein the LPAAT is present in the form of membrane vesicles.

The assay may be conducted in the presence of other
20 compounds, for example a cytokine and/or an inflammatory agent may be present.

The present invention further provides an isolated polypeptide having LPAAT activity, which has the sequence
25 sequence shown in SEQ ID NO: 2 or a sequence substantially homologous thereto, or a fragment of either said sequence. The present invention also provides an isolated polynucleotide encoding such a polypeptide. The polynucleotide can encode the amino acid sequence of SEQ ID
NO: 2. It can have the coding sequence of SEQ ID NO: 1.

30 SEQ ID NO: 1 sets out the DNA sequence of the human G15 gene isolated by the present inventors. The coding sequence runs from nucleotide 110 to nucleotide 958. The amino acid sequence of the LPAAT protein is shown in SEQ ID NOS: 1 and 2. The term "human LPAAT" refers not only to
35 the polypeptide of the invention shown in SEQ ID NO: 2 but also includes variants of this sequence with human LPAAT

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function. The polynucleotide may be incorporated into a recombinant vector such as an expression vector.

A polypeptide of the invention can be prepared according to the invention by a process comprising (i) cultivating a host cell transformed or transfected with an expression vector according to the invention under conditions to provide for expression of the said polypeptide, and (ii) recovering the expressed polypeptide.

The present invention also provides a preparation of purified membranes vesicles which comprise LPAAT in their membranes.

DESCRIPTION OF THE FIGURES

Fig.1.- Schematic representation of the first steps of phospholipid biosynthesis.

Fig.2.- a) Nucleotide sequence of the 2045bp G15 cDNA (SEQ ID NO:1) and translation thereof (SEQ ID NO:2). The predicted amino acid sequence is shown above the nucleotide sequence. Note the poly(A) signal AATAAA (underlined) 17bp upstream of a 21bp poly(A) tail. The positions of cleavage of the two potential signal sequences are indicated with arrows, the potential transmembrane domains are shaded and the potential N-glycosylation site is in bold. b) Hydrophobicity profile of the G15 gene product generated with the program PepWindow. The two potential signal sequences (arrows) and the potential transmembrane domains (horizontal bars) are indicated.

Fig.3.- a) Percentage of amino acid identities (ID) or similarities (SIM) and b) amino acid sequence alignment of the complete amino acid sequence of the G15 gene product and LPAATs from different organisms. Mammalian: human (G15); yeast: *Saccharomyces cerevisiae* (Scerevisiae); bacteria: *Haemophilus influenzae* (Haemoph), *Salmonella typhimurium* (Salmonella), *Escherichia coli* (Ecoli),

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Neisseria gonorrhoeae (Gonocc) *Neisseria meningitidis* (Meningo); and plant: *Limnanthes alba* (Lalba), *Cocos nucifera* (Cnucifera). In a) the BestFit scores are shown. In b) the conserved potential transmembrane sequences are underlined and the potential active centre is double underlined. c) Dendrogram or tree representation that displays the clustering order used to create the alignment.

Fig.4.- Expression of the G15 gene product. a) Sf21 cells infected with G15 recombinant baculovirus (vG15Bac), AcB15R, or wild type baculovirus (Wt), were pulse labelled with 200 μ Ci/ml of 35 S-Trans-label from 24 to 27 h post-infection, and proteins present in cells and in the medium were analysed by SDS-PAGE and visualised by autoradiography. The positions of the polyhedron protein (P) and the vaccinia IL-1 β receptor (.) are shown. b) Western blot analysis of cell extracts from the cell lines CHOG15, CHOV or CHO using T7.TagmAb. The proteins and the molecular size markers are indicated in kDa.

Fig.5.- Predicted topology of hLPAAT with the conserved regions in grey.

Fig.6.- LPAAT activity of the G15 gene product (hLPAAT). a) and b) Spectrophotometric assays of 50 μ g and c) TLC assays of 4 μ g of cell extracts from wt or vG15Bac baculovirus, using 10 μ M (a) or 45 μ M (b and c) of saturated or unsaturated acylCoAs: palmitoleoyl (C16:1), arachidonoyl (C20:4), linolenoyl (C18:3), linoleoyl (C18:2), oleoyl (C18:1), stearoyl (C18:0), lignoceroyl (C24:0), arachidoyl (C20:0), palmitoyl (C16:0), myristoyl (C14:0), and lauroyl (C12:0). The positions of PA and LPA are indicated.

Fig.7.- TLC assay of 4 μ g of cell extracts from wt or vG15Bac baculovirus using 100 μ M of oleoylCoA (C18:1), and 50 or 250 μ M of LPA. Blank is the reaction performed without cell extract. The positions of PA and LPA are indicated.

Fig.8.- a) Spectrophotometric assay of 150 μ g of cell extracts from the cell lines CHO (O), CHOV(\oplus) and CHOG15 (\bullet)

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using 45 μ M of oleoylCoA (C18:1). b) TLC assay of 4 or 20 μ g of cell extracts from the same cell lines using 45 μ M of linoleoylCoA (C18:2). The positions of PA and LPA are indicated. c) Total nmols of PA obtained by spectrophotometric assay after 6 min of reaction using as substrate 45 μ M of oleoylCoA and 50 and 150 μ g of cell extracts.

Fig.9.-Immunolocalisation of hLPAAT. a) CHOG15 cells labelled using the T7.TagmAb [1/150 (v/v) dilution]. The cell lines CHOG15 b), CHO c), and CHOV d), labelled with the mAb to PDI [1/60 (v/v) dilution] located in the ER. CHOV or CHO cells labelled with the T7.TagmAb did not show any staining (data not shown). Immunofluorescence was observed using a Bio Rad 1024 confocal microscope. The bar represents 10 μ m.

Fig.10.- Immunolocalisation of hLPAAT in transiently transformed Cos7 cells. A), C) Permeabilised cells and B) non permeabilised cells using the T7.TagmAb [1/3000 (v/v) dilution]. C) ER indicates cells labelled with the mAb to DPI [1/30 (v/v) dilution] located in the ER and (V3) are cells transfected with pcDNA3. The numbers in brackets indicate the day post-transfection when the cells were processed. The bar represents 25 μ m.

Fig.11. - Inhibitory effect of pentoxifylline (PTX) and lisofylline on hLPAAT activity. Spectrophotometric assay of cell extracts from vG15Bac (G15) or wild type (WT) baculovirus. The different amounts of PTX or lisofylline used are indicated. In (a) no preincubation with PTX was performed. In (b) and (c) the cell extracts were preincubated for 1 hour with different concentrations of PTX (b) or lisofylline (c) before the spectrophotometric readings were taken.

DETAILED DESCRIPTION OF THE INVENTION

35

A. Polynucleotides.

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A polynucleotide of the invention is capable of hybridising selectively with the coding sequence of SEQ ID NO: 1 or to the sequence complementary to that coding sequence. Polynucleotides of the invention include
5 variants of the coding sequence of SEQ ID NO: 1 which encode polypeptides which have LPAAT activity. Typically, a polynucleotide of the invention is a contiguous sequence of nucleotides which is capable of selectively hybridizing to the coding sequence of SEQ ID. No 1 or to the complement
10 of that coding sequence.

A polynucleotide of the invention and the coding sequence of SEQ ID NO: 1 can hybridize at a level significantly above background. Background hybridization may occur, for example, because of other cDNAs present in a
15 cDNA library. The signal level generated by the interaction between a polynucleotide of the invention and the coding sequence of SEQ ID NO: 1 is typically at least 10 fold, preferably at least 100 fold, as intense as interactions between other polynucleotides and the coding
20 sequence of SEQ ID NO: 1. The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with ³²P. Selective hybridisation is typically achieved using conditions of medium to high stringency (for example 0.03M sodium chloride and 0.03M sodium citrate at from
25 about 50°C to about 60°C).

A nucleotide sequence capable of selectively hybridizing to the DNA coding sequence of SEQ ID NO: 1 or to the sequence complementary to that coding sequence will be generally at least 70%, preferably at least 80 or 90%
30 and more preferably at least 95%, homologous to the coding sequence of SEQ ID NO: 1 or its complement over a region of at least 20, preferably at least 30, for instance at least 40, 60 or 100 or more contiguous nucleotides.

Any combination of the above mentioned degrees of
35 homology and minimum sizes may be used to define polynucleotides of the invention, with the more stringent

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combinations (i.e. higher homology over longer lengths) being preferred. Thus for example a polynucleotide which is at least 80% homologous over 25, preferably over 30 nucleotides forms one aspect of the invention, as does a
5 polynucleotide which is at least 90% homologous over 40 nucleotides.

Polynucleotides of the invention may comprise DNA or RNA. They thus consist essentially of DNA or RNA encoding the amino acid sequence of SEQ ID NO: 2. They can consist
10 essentially of the coding sequence of SEQ ID NO: 1. The polynucleotides may be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to polynucleotides are known in the art. These include methylphosphonate and
15 phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art. Such
20 modifications may be carried out in order to enhance the *in vivo* activity or lifespan of polynucleotides of the invention.

Polynucleotides of the invention may be used to produce a primer, e.g. a PCR primer, a primer for an
25 alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably
30 at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term polynucleotides of the invention as used herein.

Polynucleotides such as a DNA polynucleotide and primers according to the invention may be produced
35 recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by

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standard techniques. The polynucleotides are typically provided in isolated and/or purified form.

In general, primers will be produced by synthetic means, involving a step wise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Longer polynucleotides will generally be produced using recombinant means, for example using PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15-30 nucleotides) to a region of the LPAAT gene which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from a human cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

Such techniques may be used to obtain all or part of the G15 gene sequence described herein. Genomic clones containing the G15 gene and its introns and promoter regions may also be obtained in an analogous manner, starting with genomic DNA from a human cell, e.g. a liver cell.

Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook et al, 1989.

Polynucleotides which are not 100% homologous to the sequences of the present invention but fall within the scope of the invention can be obtained in a number of ways.

Other human allelic variants of the human LPAAT sequence of SEQ ID NO: 1 may be obtained for example by probing genomic DNA libraries made from a range of

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individuals, for example individuals from different populations, or individuals with different types of inflammatory disorders or other diseases such as diabetes.

In addition, other animal, particularly mammalian (e.g. mice or rabbits), more particularly primate, homologues of G15 may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridizing to the coding sequence of SEQ ID NO: 1 or its complement. Such sequences may be obtained by probing cDNA libraries made from dividing cells or tissues or genomic DNA libraries from other animal species, and probing such libraries with probes comprising all or part of the coding sequence of SEQ ID NO: 1 under conditions of medium to high stringency (for example 0.03M sodium chloride and 0.03M sodium citrate at from about 50°C to about 60°C).

Allelic variants and species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences. Conserved sequences can be predicted from aligning the LPAAT amino acid sequence with that of other LPAAT sequences. The primers will contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

Alternatively, such polynucleotides may be obtained by site directed mutagenesis of the G15 gene sequence or allelic variants thereof. This may be useful where for example silent codon changes are required to sequences to optimise codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction enzyme recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides.

The invention further provides double stranded

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polynucleotides comprising a polynucleotide of the invention and its complement.

Polynucleotides or primers of the invention may carry a revealing label. Suitable labels include radioisotopes
5 such as ^{32}P or ^{35}S , enzyme labels, or other protein labels such as biotin. Such labels may be added to polynucleotides or primers of the invention and may be detected using techniques known *per se*.

Polynucleotides or primers of the invention or
10 fragments thereof labelled or unlabelled may be used by a person skilled in the art in nucleic acid-based tests for detecting or sequencing polynucleotides encoding LPAAT in a sample taken from a human or animal.

Such tests for detecting generally comprise bringing
15 a human or animal sample containing DNA or RNA into contact with a probe comprising a polynucleotide or primer of the invention under hybridizing conditions and detecting any duplex formed between the probe and nucleic acid in the sample. Such detection may be achieved using techniques
20 such as PCR or by immobilizing the probe on a solid support, removing nucleic acid in the sample which is not hybridized to the probe, and then detecting nucleic acid which has hybridized to the probe. Alternatively, the sample nucleic acid may be immobilized on a solid support,
25 and the amount of probe bound to such a support can be detected. Suitable assay methods of this and other formats can be found in for example WO89/03891 and WO90/13667.

Tests for sequencing LPAAT genes include bringing a human or animal sample containing target DNA or RNA into
30 contact with a probe comprising a polynucleotide or primer of the invention under hybridizing conditions and determining the sequence by, for example the Sanger dideoxy chain termination method (see Sambrook et al, 1989).

Such a method generally comprises elongating, in the
35 presence of suitable reagents, the primer by synthesis of a strand complementary to the target DNA or RNA and

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selectively terminating the elongation reaction at one or more of an A, C, G or T/U residue; allowing strand elongation and termination reaction to occur; separating out according to size the elongated products to determine the sequence of the nucleotides at which selective termination has occurred. Suitable reagents include a DNA polymerase enzyme, the deoxynucleotides dATP, dCTP, dGTP and dTTP, a buffer and ATP. Dideoxynucleotides are used for selective termination.

The probes of the invention may conveniently be packaged in the form of a test kit in a suitable container. In such kits the probes may be bound to a solid support where the assay format for which the kit is designed requires such binding. The kit may also contain suitable reagents for treating the sample to be probed, hybridizing the probe to nucleic acid in the sample, control reagents, instructions, and the like.

The present invention also provides polynucleotides encoding the polypeptides of the invention described below. Because such polynucleotides will be useful as sequences for recombinant production of polypeptides of the invention, it is not necessary for them to be selectively hybridizable to the coding sequence of sequence SEQ ID NO: 1, although this will generally be desirable. Otherwise, such polynucleotides may be labelled, used, and made as described above if desired. Polypeptides of the invention are described below.

Particularly preferred polynucleotides of the invention are those derived from the region encoding the putative mature LPAAT protein, its allelic variants and species homologues. The mature LPAAT protein is predicted to consist of amino acid residues 59 to 283 of SEQ ID NO: 2 and is encoded by nucleotides 283 to 958 of SEQ ID NO: 1. Polynucleotides of the invention which comprise at least this region are particularly preferred. Also preferred are polynucleotides which encode amino acids 23 to 283 of SEQ

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ID NO: 1 and amino acids 1 to 283.

An additional class of polynucleotides of the invention which are preferred are those which encompass nucleotides 635-697 corresponding to amino acids 176-196 of
5 SEQ ID NO: 1. This region is believed to represent the active centre of LPAAT. Thus this sequence will be a useful probe sequence and polypeptides encoded by this sequence may be of use in studying the function of LPAAT and in the design of inhibitor molecules. Thus fragments
10 of the above mentioned larger preferred nucleotide sequences of the invention which include the 635-697 region are preferred.

Polynucleotides and primers of the invention can be used for looking at polymorphism in the LPAAT gene
15 including regions around this gene such as regulatory regions including the 5' promoter regions. Individuals with disease states such as diabetes and rheumatoid arthritis can be examined for gene polymorphism and this can be examined for correlation with disease status.

20 In addition conserved regions of the LPAAT DNA sequence (in particular the potential active centre) can be used to identify LPAAT from other organisms or new acyltransferase that may have other different affinities for acylCoAs producing distinct PAs involved in the signal
25 transduction pathways activated by other stimuli.

B. Polypeptides.

A polypeptide of the invention consists essentially
30 of the amino acid sequence set out in SEQ ID NO: 2 or a substantially homologous sequence, or of a fragment of either of these sequences. In general, the naturally occurring human LPAAT amino acid sequence shown in SEQ ID NO: 2 is preferred. However, the polypeptides of the
35 invention include homologues of the natural LPAAT sequence, and fragments of the natural LPAAT sequence and of its

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homologues, which are capable of functioning as an LPAAT.
In particular, a polypeptide of the invention may comprise:

- (a) the protein of SEQ ID No: 2; or
- (b) an allelic variant or species
5 homologue thereof; or
- (c) a protein at least 70% homologous to (a) or
 (b).

An allelic variant will be a variant which will occur
naturally in a human and which will function in a
10 substantially similar manner to the protein of SEQ ID NO:
2, for example it will be involved in signal transduction
and inflammation. Similarly, a species homologue of the
protein will be the equivalent protein which occurs
naturally in another species and which performs the
15 equivalent function in that species to the LPAAT. Such a
homologue may occur in animals such as mammals (e.g. mice,
rats or rabbits), especially primates. Within any one
species, a homologue may exist as several allelic variants,
and these will all be considered homologues of the protein
20 of SEQ ID NO: 2.

Allelic variants and species homologues can be
obtained by following the procedures described herein for
the production of the polypeptide of SEQ ID NO: 2 and
performing such procedures on a suitable cell source, eg
25 from a rodent carrying an allelic variant or another
species. It will also be possible to use a G15 nucleotide
sequence to probe libraries made from rodent or other cells
in order to obtain clones encoding the allelic or species
variants. The clones can be manipulated by conventional
30 techniques to identify a polypeptide of the invention which
can then be produced by recombinant or synthetic techniques
known *per se*. Preferred species homologues include
mammalian species homologues.

A protein at least 70% homologous to the protein of
35 SEQ ID NO: 2 or an allelic variant or species homologue
thereof will be preferably at least 80 or 90% and more

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preferably at least 95%, 97% or 99% homologous thereto, for example over a region of at least 20, preferably at least 30, for instance at least 40, 60 or 100 or more contiguous amino acids. Methods of measuring protein homology are well known in the art and it will be understood by those of skill in the art that in the present context, homology is calculated on the basis of amino acid identity (sometimes referred to as "hard homology").

The sequence of the protein of SEQ ID NO: 2 and of allelic variants and species homologues can thus be modified to provide polypeptides of the invention.

Polypeptides of the invention include polypeptides in substantially isolated form which comprise the sequence set out in SEQ ID NO: 2.

Polypeptides further include variants of such sequences, including naturally occurring allelic variants and synthetic variants which are substantially homologous to said polypeptides. In this context, substantial homology is regarded as a sequence which has at least 70%, e.g. 80% or 90% amino acid homology (identity) over 30 amino acids with the sequence of SEQ ID NO: 2.

Polypeptides also include those encoding LPAAT homologues from other species including animals such as mammals (e.g. mice, rats or rabbits), especially primates, and variants thereof as defined above.

Polypeptides of the invention also include fragments of the above mentioned full length polypeptides and variants thereof, including fragments of the sequence set out in SEQ ID NO: 2.

Amino acid substitutions may be made, for example from 1, 2 or 3 to 10, 20 or 30 substitutions. The modified polypeptide generally retains LPAAT activity. Conservative substitutions may be made, for example according to the following Table. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

Polypeptides of the invention also include fragments
 5 of the above-mentioned full length polypeptides and
 variants thereof, including fragments of the sequence set
 out in SEQ ID NO: 2. Such fragments can retain LPAAT
 activity.

Preferred fragments include those which include an
 10 epitope. Suitable fragments will be at least about 5, e.g.
 10, 12, 15 or 20 amino acids in size. Polypeptide fragments
 of the LPAAT protein and allelic and species variants
 thereof may contain one or more (e.g. 2, 3, 5, or 10)
 15 substitutions, deletions or insertions, including conserved
 substitutions. Epitopes may be determined either by
 techniques such as peptide scanning techniques as described
 by Geysen et al, 1986.

Polypeptides of the invention may be in a
 substantially isolated form. It will be understood that
 20 the polypeptide may be mixed with carriers or diluents
 which will not interfere with the intended purpose of the
 polypeptide and still be regarded as substantially
 isolated. A polypeptide of the invention may also be in a
 substantially purified form, in which case it will
 25 generally comprise the polypeptide in a preparation in
 which more than 90%, e.g. 95%, 98% or 99% of the
 polypeptide in the preparation is a polypeptide of the
 invention.

Polypeptides of the invention may be modified for
 30 example by the addition of Histidine residues or a T7 tag

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to assist their identification or purification or by the addition of a signal sequence to promote their secretion from a cell where the polypeptide does not naturally contain such a sequence.

5 A polypeptide of the invention may be labelled with a revealing label. The revealing label may be any suitable label which allows the polypeptide to be detected. Suitable labels include radiosotopes, e.g. ^{125}I , ^{35}S , enzymes, antibodies, polynucleotides and linkers such as
10 biotin. Labelled polypeptides of the invention may be used in diagnostic procedures such as immunoassays in order to determine the amount of a polypeptide of the invention in a sample.

Polypeptides or labelled polypeptides of the
15 invention may also be used in serological or cell mediated immune assays for the detection of immune reactivity to said polypeptides in animals and humans using standard protocols. The labelled polypeptide may be used to identify and/or isolate "accessory" proteins which are
20 involved between cell receptors and LPAAT, by detecting the interaction of LPAAT to such proteins.

A polypeptide or labelled polypeptide of the invention or fragment thereof may also be fixed to a solid phase, for example the surface of an immunoassay well or
25 dipstick.

Such labelled and/or immobilized polypeptides may be packaged into kits in a suitable container along with suitable reagents, controls, instructions and the like. Kits may be used for LPAAT activity screening assays. The
30 kits may also be used to identify LPAAT inhibitors or activators. The kits may also contain suitable reagents in which to carry out the activity assays, for example LPA and/or acyl-CoA.

Such polypeptides and kits may also be used in
35 methods of detection of antibodies to the LPAAT protein or its allelic or species variants by immunoassay.

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Immunoassay methods are well known in the art and will generally comprise:

- 5 (a) providing a polypeptide comprising an epitope bindable by an antibody against said protein;
- (b) incubating a biological sample with said polypeptide under conditions which allow for the formation of an antibody-antigen complex; and
- 10 (c) determining whether antibody-antigen complex comprising said polypeptide is formed.

Polypeptides of the invention may be made by synthetic means (e.g. as described by Geysen *et al*) or
15 recombinantly, as described below.

Particularly preferred polypeptides of the invention include those comprising the putative mature LPAAT of SEQ ID NO: 2 from amino acid residues 59 to 283.

Other preferred polypeptides are those comprising
20 amino acids 23-283 and 1-283 of SEQ ID NO: 2.

An additional group of preferred polypeptides of the invention are those comprising the potential conserved active centre corresponding to amino acids 176-196 of SEQ ID NO: 2. Fragments which comprise this portion of LPAAT
25 and sufficient N- and C- terminal sequences to provide a polypeptide retaining LPAAT activity are also preferred. The amount of N- and C- terminal residues in addition to the conserved region may be identified by routine screening methods which involve making N- and C- terminal deletions
30 to the mature polypeptide and determining whether those deletions result in a product which retains LPAAT activity.

The polypeptides of the invention may be introduced into a cell by *in situ* expression of the polypeptide from a recombinant expression vector. The expression vector
35 optionally carries an inducible promoter to control the expression of the polypeptide.

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Such cell culture systems in which polypeptides of the invention are expressed may be used in assay systems.

A polypeptide of the invention can be produced in large scale following purification by HPLC or other
5 techniques after recombinant expression as described below. Large scale production will allow the structure of the enzyme to be elucidated to allow rational drug design.

C. Vectors.

10

Polynucleotides of the invention can be incorporated into a recombinant replicable vector. A vector of the invention consists essentially of a polynucleotide of the invention, therefore. The vector may be used to replicate
15 the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and
20 growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells are described below in connection with expression vectors.

25 D. Expression Vectors

Preferably, a polynucleotide of the invention in a vector is operably linked to a control sequence which is capable of providing for the expression of the coding
30 sequence by the host cell, i.e. the vector is an expression vector. Such expression vectors can be used to express the LPAAT for the assays of the invention.

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship
35 permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is

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ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

Such vectors may be transformed into a suitable host cell as described above to provide for expression of a polypeptide or polypeptide fragment of the invention. Thus, in a further aspect the invention provides a process for preparing polypeptide or polypeptide fragment according to the invention, which process comprises cultivating a host cell transformed or transfected with an expression vector as described above under conditions to provide for expression of the polypeptide or fragment, and recovering the expressed polypeptide or fragment.

The vectors may be for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell. The vector may also be adapted to be used *in vitro*, for example in a method of gene therapy.

A further embodiment of the invention provides host cells transformed or transfected with the vectors for the replication and expression of polynucleotides of the invention. The cells will be chosen to be compatible with the said vector and may for example be bacterial, yeast, insect or mammalian.

Polynucleotides according to the invention may also be inserted into the vectors described above in an antisense orientation in order to provide for the production of antisense RNA. Antisense RNA or other antisense polynucleotides may also be produced by synthetic means. Such antisense polynucleotides may be used in a

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method of controlling the levels of LPAAT or its variants or species homologues.

Promoters and other expression regulation signals may be selected to be compatible with the host cell for which the expression vector is designed. For example, yeast promoters include *S. cerevisiae* GAL4 and ADH promoters, *S. pombe* nmt1 and adh promoters. Mammalian promoters include the metallothionein promoter which can be induced in response to heavy metals such as cadmium. Most preferably, the expression vectors are possible for use in insect or mammalian cells. For use in insect cells, strong baculovirus promoters such as the polyhedron promoter are preferred. For expression in mammalian cells, strong viral promoters such as the SV40 large T antigen promoter, a CMV promoter or an adenovirus promoter may also be used. All these promoters are readily available in the art.

20

E. Expression in host cells

Expression vectors of the invention may be introduced into host cells using conventional techniques including calcium phosphate precipitation, DEAE-dextran transfection, or electroporation. Expression from the host cell may be transient. In this situation the assay of the invention will be performed shortly after transfection for example from 1 to 96 hours after transfection. Where LPAAT is to be recovered in the form of vesicles containing LPAAT, the later end of this range is preferred, for example within from 24 to 96 hours of transfection.

The expression vector may contain a selectable marker and/or such a selectable marker may be co-transfected with the expression vector and stable transfected cells may be selected. Pools of transfected cells may be obtained or

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single cell clones of transfectants may be obtained and screened for levels of LPAAT activity.

Suitable cells include cells in which the abovementioned vectors may be expressed. These include
5 microbial cells such as bacteria such as *E. coli*, mammalian cells such as CHO cells, COS7 cells, P388 cells, HepG2 cells, KB cells, EL4 cells or Hela cells, insect cells or yeast such as *Saccharomyces*. Baculovirus or vaccinia expression systems may be used.

10 Cell culture will take place under standard conditions. Commercially available cultural media for cell culture are widely available and can be used in accordance with manufacturers instructions.

15 F. Antibodies

The invention also provides monoclonal or polyclonal antibodies which bind to polypeptides of the invention. Such monoclonal antibodies may be prepared by conventional
20 hybridoma technology using polypeptides of the invention as immunogens. Polyclonal antibodies may also be prepared by conventional means which comprise inoculating a host animal, for example a rat or a rabbit, with a polypeptide of the invention and recovering immune serum. In order
25 that such antibodies may be made, polypeptides may be haptenised to another polypeptide for use as immunogens in animals or humans. For the purposes of this invention, the term "antibody" includes antibody fragments such as Fv, F(ab') and F(ab')₂ fragments, as well as single chain
30 antibodies. Furthermore, the antibodies and fragments thereof may be humanised antibodies, e.g. as described in EP-A-239400.

The antibodies of the invention are thus specific for the polypeptides of the invention, for example the LPAAT
35 protein of SEQ ID NO: 2. The antibodies may be used for detecting polypeptides of the invention present in

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biological samples by a method which comprises:

- (a) providing an antibody of the invention;
- 5 (b) incubating a biological sample with said antibody under conditions which allow for the formation of an antibody-antigen complex; and
- 10 (c) determining whether antibody-antigen complex comprising said antibody is formed.

Suitable samples include extracts from brain tissue, both normal and neoplastic. Suitable samples may also include extracts from other tissues such as breast, ovary, lung, colon, pancreas, testes, liver, muscle and bone
15 tissues or from neoplastic growths derived from such tissues.

Antibodies of the invention may be bound to a solid support and/or packaged into kits in a suitable container along with suitable reagents, controls, instructions and
20 the like.

G. Assay methods.

The assay of the invention may be performed using any
25 suitable conditions in which the recombinant LPAAT can function and its activity can be measured.

In its simplest form, the LPAAT is expressed in a host cells and a culture of host cells is homogenised in a buffer such as a Tris-HCl and or Hepes buffer at around pH
30 7.0 to 8.0. The homogenate may be used directly or fractionated to partially or completely purify the fraction containing LPAAT. For example when the LPAAT is expressed in eukaryotic cells, particularly mammalian cells, the cell extract may be used directly or fractionated so as to
35 retain the microsomal fraction which contains LPAAT activity. Such fractionation is usually by centrifugation,

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e.g. at a low speed (1,000 to 2,000 rpm) for 5 to 20 minutes to remove debris, optionally and desirably followed by a high speed centrifugation (e.g. 10,000 to 15,000 rpm for 15 to 60 minutes) to pellet the microsomal fraction which is then resuspended.

In general, cell homogenisation and fractionation techniques aimed at recovering cell proteins in active form are well known. Details of such techniques may be found in, for example, *Molecular Biology of the Cell*, 2nd Edn, Alberts, B., et al, 1989.

We have also found that when LPAAT is expressed at high levels in COS7 cells (African Green monkey cells transformed with the virus SV40) these cells produce membrane vesicles in which the LPAAT is localised in the membrane. While not wishing to be bound by any one theory, it is known that lysophosphatidic acid-containing vesicles and secretory PLA₂ have been detected in inflammatory fluids of patients with rheumatoid arthritis (Fourcade et al., 1995). Thus these patients could be overexpressing the hLPAAT and secreting these vesicles, and this could contribute to their inflammatory response, where the PLA₂ has been induced to block the overproduction of PA resulting in the generation of LPA. In view of this the production of LPAAT in vesicles may be a desirable mode for the assay of the invention since it may reflect the environment in which LPAAT is produced in patients in which inhibition of the enzyme could be beneficial.

Another advantage of this mode of assay is to provide the enzyme still in a membrane bound state but in a less complex environment than in a whole cell. This may reduce the amount of or remove entirely cellular components which may interfere with or affect the outcome of the assay.

Although in the accompanying examples we have found vesicles to be produced in COS7 cells, other cells in which overexpression of LPAAT causes vesicle production to occur

- 30 -

may be used, including other mammalian cells including human, primate or rodent cells. The cells may be transfected with an expression vector of the invention (see below) and examined to ascertain whether the combination of cell type and expression vector provides for vesicle production.

The substrate used in the assay of the invention is any lysoPA substrate for LPAAT. This may be naturally occurring or synthetic. Preferably the fatty acyl chain at the *sn*-1 position desirably contains from 12 to 24 carbon atoms, for example from 14 to 20 carbon atoms, and may be saturated or may contain from 1 to 4 double bonds. Preferably the fatty acyl chain is a C₁₈ chain such as oleoyl which contains a single double bond.

The amount of substrate in the assay will be determined by the reaction conditions used and the scale on which it is performed. A concentration of from about 1 μ M to about 1000 μ M will be suitable, e.g from about 10 μ M to 100 μ M.

The cofactor in the assay will be a cofactor which comprises a fatty acid group which will be attached to LPA at the *sn*-2 position by the action of LPAAT. This will generally be a fatty acid acyl-coenzyme A compound or other fatty acid compounds found in both normal and unusual inflammatory responses. The compound will preferably have a fatty acid chain of from 12 to 24 carbon atoms, more preferably from 16 to 24 carbon atoms which is either saturated or contains from 1 to 4 double bonds. Most preferably the cofactor comprises a linoleoyl, oleoyl or arachidonoyl group.

The amount of cofactor used in the assay will be dependent to a large extent on the concentration of lysoPA, and will usually be in a ratio of from 1:10 to 10:1, preferably from 1:5 to 1:1 of cofactor to substrate.

The activity of the LPAAT may be determined in any suitable way. In general this will be by measuring the

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disappearance of the substrate or cofactor or the appearance of PA. In the case of the cofactor, removal of the fatty acyl chain will leave a free thiol group on the released coenzyme A, and the presence of this free thiol group may be measured, for example as described in the accompanying examples. Alternatively the LPA or the acyl chain of the cofactor may be labelled with a radioisotope such as ^{14}C or ^3H and the fate of the label be determined. Suitable methods for determining the fate of the label include chromatography such as thin layer chromatography as described in the accompanying examples.

The candidate inhibitor may be any compound suspected to be of use in inhibiting the inflammatory response via the action of LPAAT activity. Thus the inhibitor may be something which inhibits the activation of LPAAT by interfering with a signalling pathway for the enzyme or it may be an inhibitor which directly blocks, alters or affects the catalytic function of the enzyme. It would be appreciated by those of skill in the art that any part of the inflammation pathway mediated via LPAAT can be assayed in accordance with the present invention since disruption of the signalling pathway preventing LPAAT being activated is potentially as effective as directly inhibiting the catalytic activity of the enzyme itself. In any event, putative inhibitors which may be considered for use in the assay include, but are not limited to, naturally occurring compounds or mixtures thereof (for example in the form of plant extracts), peptides and synthetic chemicals. Peptides may include fragments of the LPAAT polypeptide shown in SEQ ID NO:1, e.g peptides of from 8 to 20 amino acids. Synthetic chemicals may include xanthine derivatives such as the derivatives of type described in WO95/13075. Inhibitors can also be synthetic modified fatty acids that could compete with the naturally occurring fatty acids.

Candidate inhibitors will desirably be added to the

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assay at concentrations of, say from 0.1 nM to 1000 μ M, e.g. 10 nM to 100 μ M, preferably from 100 nM to 10 μ M.

In a further embodiment of the invention, the assay may be conducted in the presence of one or more (e.g. two or three) cytokines and/or one or more (e.g. two or three) inflammatory agents, particularly a cytokine such as an interleukin (e.g. IL-1 (such as IL-1 β), IL-6, IL-2) interferons (e.g. IFN α), chemokines (e.g. IL-8), or TNF α / β which are known to be mediators of the inflammatory response. This format of assay may be used for example to characterise further those candidate inhibitors which show positive LPAAT inhibitory activity in the assay of the invention.

Where the assay is conducted in the presence of a cytokine it will be desirable to use a cell or extract thereof which has been shown, by prior routine testing, to contain receptors for the cytokine which trigger an increase in the production of an inflammatory response, preferably by an increase in synthesis of PA. In such an event the cell extract will be compatible with the cytokine being used. In some cases, cytokines from one species will be active in cells from another species. Otherwise, the cytokine used will be derived from the species corresponding to the cell type or the cells of the assay will be modified to express a cytokine receptor compatible with the cytokine used.

The amount of each cytokine used will depend upon its nature. For example, IL-1 can be used at around 1nM to 100 μ M whereas TNF α can be used at around 10pg/ml to 500ng/ml.

Other inflammatory agents such as LPS or the major active component of bacterial endotoxins, lipid A, may also be used. LPS can be used at a concentration of around 1-100ng/ml, and lipid A at 0.01ng/ml - 1000ng/ml.

In addition to the assay described above, the present invention may also be used to define LPAAT activators

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responsible for causing sepsis or inflammation. Thus putative activators may be used in exactly the same manner as putative inhibitor compounds described above. Such putative activators may include previously uncharacterised molecules such as polypeptides found in human serum.

Thus in this aspect of the invention the assay may be used against compounds suspected of being activators of the inflammatory response or biological samples containing otherwise uncharacterised factors which mediate an immune response. Such biological samples may be, for example, serum from a patient with an inflammatory condition, such as rheumatoid arthritis. The biological sample may be fractionated, for example, by chromatography to identify individual fractions which are active in the assay of the invention. Thus the invention provides a means to identify further mediators of the inflammatory response. Moreover, putative inhibitor compounds can be used in assays of the invention in the presence of these newly identified mediators to identify specific inhibitor compounds for such mediators.

The effect of a test substance on LPAAT activity may be measured in two or more different types of assay. It may be desired that the test substance inhibits LPAAT activity in one type of assay and does not inhibit LPAAT activity in another type of assay. A test substance may, for example, only inhibit the increase in LPAAT activity caused by stimulation with an inflammatory agent. To determine whether a test substance has such a property the effect of the substance on LPAAT activity can be measured both in:

- i) an assay wherein the LPAAT is present in an extract from cells which have been contacted with an inflammatory agent; and
- ii) an assay wherein the LPAAT is present in an extract from cells which have not been contacted with an inflammatory agent.

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The cells of (i) may be of the same type or of a different type from the cells of (ii). The cells of (i) may be mammalian cells such as mouse or human cells. They may be P388 cells. The inflammatory agent may be LPS, IL-1, lipid A, or TNF- α . The cells of (ii) may be cells in which that particular LPAAT protein would not be naturally expressed such as non-mammalian cells expressing a mammalian LPAAT, such as a human or mouse LPAAT. Such cells may be those which are conventionally used to express recombinant proteins, such as microbial cells or insect cells. The cells may be Sf21 insect cells in which the mammalian LPAAT is expressed using a baculovirus expression system.

A test substance may for example inhibit LPAAT activity by affecting LPAAT activation pathways. To determine whether a test substance has such a property the effect of the test substance on LPAAT activity could be measured both in:

- i) an assay wherein the LPAAT is present in an extract from a cell type in which that LPAAT would naturally be expressed; and
- ii) an assay wherein the LPAAT is present in an extract from cells in which the LPAAT has been expressed recombinantly, and in which cells that particular LPAAT protein would not naturally be expressed. For instance the recombinant LPAAT may be from a different species than the species from which the cell is from.

In addition the assay of the invention can be used to examine new enzyme stimuli such as cytokines, and whether the stimuli are acting independently or in combination or by stimulating another intermediate protein which activates LPAAT. Different cells expressing particular receptors might be transfected with the LPAAT sequence of the invention in order to see whether it is possible to induce the enzyme with different stimuli, or whether there is a

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need for an intermediate protein to elucidate the effect of particular inhibitors.

In a further aspect of the invention the LPAAT enzyme may be expressed and used to synthesise a phosphatidic acid (PA). The PA may subsequently be purified by HPLC. This
5 may be particularly advantageous since PAs are difficult to obtain and thus isolation of them can be used to study in m role in inflammation.

The physical format of the assay of the invention may
10 be determined by those of skill in the art taking into account factors such as the way in which LPAAT is expressed, the nature of the inhibitor compounds being screened and the degree of quantification required from the results. A suitable assay format includes the use of
15 microtiter plates (e.g. 12, 48 or 96-well plates) in which the wells of the plate contain cell extracts containing LPAAT. The necessary co-factor and substrate compound are added in the presence and absence of the inhibitor compound. The mixture is then incubated for a suitable
20 period of time (for example 1 second - 20 minutes, e.g. 1 min - 10 min). Where the results of the assay is to be read by a colormetric change (as described in the examples below) the necessary colour-change reagents may also be added to the assay. Alternatively, when the assay format
25 relies on the incorporation of label into a specific product the wells may be sampled and the samples obtained from the wells subjected to chromatography such as TLC or HPLC as described above. Optionally lipids can be extracted from the samples and the lipids subjected to the
30 mentioned chromatographic analysis.

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The following examples further illustrate the invention

EXAMPLE 1

5

Nucleotide sequence of G15 cDNA

The G15 gene, which is localised in the class III region of the MHC ~260kb telomeric of the DRA gene. G15 is
10 a single copy gene as defined by comparative analysis of genomic and cosmid Southern blots. Northern blot analysis of RNA from the cell lines U937 (promonocytic leukaemia), Molt4 (T-cell leukaemia) and Raji (Burkitt lymphoma) revealed that the G15 gene encodes a 2.1kb mRNA (Kendall et
15 al., 1990).

Screening of a U937 cDNA library, using two overlapping cosmids (D3A and E91) from the MHC class III region as probes, resulted in the isolation of 22 cDNA clones. Characterisation of these clones by restriction
20 enzyme mapping revealed that pG15-3B contained a full-length cDNA insert of ~2.1kb (Kendall et al., 1990). Both strands of this cDNA were sequenced by the dideoxy chain termination method after cloning random sonicated fragments in the size range 300-1000bp by blunt end ligation into
25 SmaI-cut M13mp18. The sequence was assembled using Staden (Staden, 1987) programs and determined with a degeneracy of 9.0. Computer analyses were performed using the software package of the University of Wisconsin Genetics Computer Group (GCG) (Pearson and Lipman, 1988). The insert of pG15-
30 3B was found to be a 2045 bp sequence containing a poly(A) signal AATAAA 17bp upstream of a 21bp poly(A) tail. This sequence is shown in SEQ ID NO:1 and includes the coding sequence of the G15 gene.

Translation of the DNA sequence in different phases
35 revealed a single long open reading frame which was

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predicted to encode a polypeptide of 283 amino acids (Fig.2a) and with a predicted molecular weight of ~31.7kDa. The hydrophobicity plot of the G15 gene product is shown in Fig.2b indicating that the G15 gene encodes a hydrophobic protein. At the N-terminus there are two putative signal cleavage sites, after amino acids 22 and 58, and there are in total 7 potential hydrophobic regions, suggesting that the G15 gene product could be a transmembrane protein.

10 G15 gene product database search

A search of the SWISSPROT databases, using the FastA program from the GCG package, with the G15 gene product sequence, revealed homology with the entire sequence of LPAAT from different bacteria (*Haemophilus*, *Salmonella*, *Escherichia*, and *Neisseria*), from yeast (*Saccharomyces*) and from plant (*Limnantes* and *Cocos*) which suggests that the G15 gene encodes the human LPAAT (hLPAAT) (Fig.3). Using the BestFit program from the GCG package, the G15 gene product was shown to be related to the LPAAT from *Saccharomyces cerevisiae* (30.9% identity and 54.4% similarity) (Nagiec et al., 1993), from *Haemophilus influenzae* (30.4% identity and 56.7% similarity) (Fleischmann et al., 1995), from *Salmonella typhimurium* (28.1% identity and 51.2% similarity) (Luttinger et al., 1991), from *Escherichia coli* (27.7% identity and 51.2% similarity) (Coleman, 1992), from *Limnantes alba* (28.4% identity and 53.3% similarity) (Lassner et al., 1995), from *Cocos nucifera* (23.6% identity and 52.8% similarity) (Knutzon et al., 1995), and from *Neisseria gonorrhoeae* (24.9% identity and 47.2% similarity) and *Neisseria meningitidis* (24.0% identity and 47.6% similarity) (Swartley et al., 1995) (Fig.3a). Multiple sequence

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alignment of these sequences (PileUp program from the GCG package) shows that they are highly related (Fig.3b) and the corresponding dendrogram indicates the relationships (Fig.3c).

5

EXAMPLE 2

Expression of LPAAT in insect cells using the baculovirus system

10

To characterise the G15 gene product, the protein was expressed in *Spodoptera frugiperda* (Sf) 21 insect cells infected with baculovirus (*Autographa Californica* Nuclear Polyhedrosis Virus, AcNPV) and in the mammalian cell line CHO (Chinese hamster ovary) (Example 3).

To remove the 3' and 5' flanking sequences of the G15 cDNA, in order to express only the coding sequence under the control of the polyhedrin promoter, a PCR copy of the open reading frame was generated using oligonucleotide primers that also created *Xba*I sites, and this was ligated into the *Xba*I-digested plasmid pBluescript KS⁺ (pBlsc). Several clones were sequenced. The insert of one clone (pG15Bls) that did not include any PCR errors was excised, and ligated to the baculovirus transfer vector pAcCL29.1 (Livingstone and Jones, 1989), kindly donated by Dr. I. Jones (NERC Institute of Virology and Environmental Microbiology, Oxford) which had been digested with *Xba*I to yield pG15Bac. Sf21 cells were cotransfected with BacPAK6 DNA (Clontech) and pG15Bac, using lipofectin (GIBCO), to create G15 recombinants. After the cotransfection and the first plaque assay, ten clones were isolated and plaque purified four times. The expression of the recombinant G15 gene product was confirmed by metabolic labelling. Sf21 cells were infected with the baculovirus wild type or

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recombinants at 10 plaque forming units/cell. 24 hours post-infection, infected and uninfected cells were pulse-labelled for three hours with 200 μ Ci/ml 35 S Trans-label (ICN Biomedicals; a mixture of ~80% [35 S]methionine and ~20% [35 S]cysteine, 1200 Ci/mmol) in methionine-free TC100 medium, in the absence of serum. Cells, or the extracellular media, were boiled in sample buffer (60mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol) and analysed by SDS-PAGE in 12% acrylamide gels as described previously. Radioactive bands were detected by fluorography with Amplify (Amersham). One of the viruses, vG15Bac, was selected for further experiments. The recombinant AcB15R has been described previously (Alcami and Smith, 1992).

Cell extracts from cells infected with vG15Bac showed a major specific polypeptide of 26kDa and a minor specific polypeptide of 28kDa that were not obtained with a control virus expressing the secreted vaccinia IL-1 β receptor (AcB15R) (Alcami and Smith, 1992) or with the wild type (wt) virus (Fig.4a). hLPAAT was not detected in the medium (Fig.4a) in contrast to the secreted control AcB15R. When the radiolabelling was performed in the presence of tunicamycin, no difference in the hLPAAT size was observed (data not shown) indicating that the single potential N-glycosylation site (NGS) situated at amino acids 184-186 (Fig.2a) is not glycosylated, consistent with its predicted cytosolic localisation (see below).

EXAMPLE 3

Expression of LPAAT in the mammalian cell lines CHO, P388 and HepG2

The G15 gene product was also expressed in the mammalian cell lines CHO (Chinese hamster ovary), P388 (mouse monocyte-macrophage) and HepG2 (human hepatocellular

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carcinoma). For expression in CHO cells, a 14 amino acid T7 epitope tag (T7.Tag) was fused to the C-terminus of hLPAAT, and stable cell lines expressing hLPAAT (CHO G15) or containing only the transfer vector pcDNA3 (CHO V) were produced. In order to obtain the G15 gene product fused at the C-terminus to a T7.Tag sequence (MASMTGGQQMGRDP), for which specific monoclonal antibodies are commercially available (T7.TagmAb) (Novagen), the last 270bp encoding the C-terminus of the coding sequence of the G15 gene product (from pG15Blsc), were PCR amplified. In this amplification a *NcoI* site at the 3' end of the cDNA was created to remove the stop codon and to make it possible to fuse hLPAAT in frame to the T7.Tag sequence, located in T7.TagpBlsc (kindly donated by C. Winchester, from this laboratory). The PCR product was isolated and cloned into pBlsc. Several clones were sequenced to choose one without PCR errors and one clone (pG15COOH-Blsc) was digested with *AvaI-NcoI* to isolate the insert. To obtain the 5' end of the coding sequence pG15Blsc was digested with *XbaI*, end filled, then digested with *AvaI* and the 0.68kb *XbaI/AvaI* fragment isolated and ligated together with the *AvaI-NcoI* 3' end fragment into *EcoRV/NcoI* digested T7.TagpBlsc. The proper ligation of the two fragments into the vector was confirmed by sequence analysis. A *HindIII-XbaI* insert was isolated containing the hLPAAT cDNA fused to the T7.Tag sequence, and this was cloned into pcDNA3 (Invitrogen) cut with *HindIII-XbaI* to generate pcDNA3G15Tag. pcDNA3 is a vector designed for high-level stable and transient expression in eukaryotic cells where expression of the encoded protein is driven by the human cytomegalovirus promoter. DNA of one of these clones (pcDNA3G15Tag), as well as the pcDNA3 vector, were prepared using the Wizard maxi/prep procedure (Promega).

CHO cells were electroporated with linearized (*SspI*)

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pcDNA3G15Tag or pcDNA3 and incubated for 2 days with GMEMS (for CHO cells, Advanced Protein Products Ltd), supplemented with 10% (v/v) foetal calf serum (FCS). After that time cells were subcultured at different densities containing different concentrations of geneticin sulphate (G418). G418 is used for the selection of eukaryotic cells stably transfected with vectors containing the neomycin resistance gene (neo), as in pcDNA3. Expression of the protein was confirmed by Western blot analysis (ECL method, Amersham) after 13 days of transfection using the T7.TagmAb [1/1500 (v/v) dilution], and those groups of cells expressing the hLPAAT recombinant protein were diluted to obtain single clones expressing hLPAAT. Expression of the protein in the single clones was confirmed by Western blotting and three of them (CHOG15.17, .22 and .14) were analysed by immunofluorescence, and for LPAAT activity. One of the clones, CHOG15.14, was chosen for subsequent experiments (CHOG15) as it was the one which, after amplification, was found to produce a higher percentage of cells expressing the recombinant protein. Single clones containing only the vector were created in parallel and the presence of the vector was confirmed by PCR screening using specific pcDNA3 primers. Three of these clones (CHOV.18, .28 and .10) were used in immunofluorescence and enzyme activity assays as negative controls for the hLPAAT transfectants, and one of these, clone CHOV.10 was chosen for further experiments (CHOV). A similar strategy was performed to stably express recombinant hLPAAT in the cell lines P388 and HepG2.

Western blot analysis of cell extracts from the cell lines CHO, CHOG15 or CHOV using a T7.Tag monoclonal antibody (mAb), showed a specific tagged hLPAAT band of 27kDa (Fig.4b). Using a similar approach the same specific band was observed in P388G15 and HepG2G15 when compared to P388, P388V, HepG2 and HepG2V (data not shown).

EXAMPLE 4

Topology of the protein

5 The SigCleave program in the GCG package predicts two
signal peptides, one that would be cleaved after amino acid
22, and the second after amino acid 58 (Fig.2). Since the
expected molecular mass for hLPAAT is ~31.7kDa including
the signal peptide, the ~28 and ~26kDa forms found in
10 insect cells could be explained by the processing of hLPAAT
after each of the potential cleavage sites. However, in
CHOG15 cells only a single band of ~27kDa was seen. When
the molecular weight of the 14 amino acid T7.Tag is taken
into account the size of the hLPAAT polypeptide will be
15 ~26kDa, and this is similar in size to the major band found
for the G15 gene product expressed in insect cells using
baculovirus (Fig.4). This indicates that the major cleavage
site for the signal peptide is at amino acid 58 and that
the cleavage at amino acid 22 observed in insect cells
20 could be due to aberrant processing. It is also possible
that anomalous migration of the protein (due to its
hydrophobic nature) during SDS-PAGE is taking place making
it difficult to estimate the true size of the polypeptide
and thus which, if any, of the potential cleavage sites is
25 used. Further experiments will be done to characterise the
amino terminus of the mature protein.

The predict-protein program (EMBL, Heidelberg) (Rost
et al., 1995), using the alignment of the different LPAATs
and assuming cleavage of the hLPAAT after amino acid 58,
30 predicts only two transmembrane domains, one from amino
acids 130 to 147, and one from amino acids 195 to 211
(Fig.2 and 3b). The predicted topology of the protein would
be as illustrated in Fig.5 with the amino-terminus in the
ER lumen, followed by a transmembrane domain, a cytoplasmic
35 loop, another transmembrane domain, and with the C-terminus

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in the ER lumen. A highly conserved region, distinct from the transmembrane regions, between amino acids 176 and 196, would be located on the cytosolic side of the ER membrane, where phospholipid synthesis occurs, indicating a potential active centre of the enzyme (Fig.3b and 5). Comparative sequence analysis of the different LPAATs with the available GPAT sequences reveals that they show a higher sequence similarity in this region, supporting the hypothesis of this being the active centre (data not shown). The two conserved transmembrane regions could also be part of the active centre, and select for the length and degree of saturation of the acyl chains (Fig.3b and 5). In this model, the potential glycosylation site would be located on the cytosolic side of the ER and for this reason would be unavailable for glycosylation. The model would still be valid for the localisation of the active centre and carboxy-terminus even if none of the cleavage sites is used, or the cleavage site after amino acid 22 is used, since in these situations the predicted topology of the protein would only be different at the amino-terminus, due to the addition of extra transmembrane domains.

EXAMPLE 6

1-Acyl-Glycerol-3-Phosphate-Acyltransferase activity of the G15 gene product

To demonstrate that the G15 gene product is the hLPAAT, the activity of the recombinant protein was shown *in vitro* by spectrophotometric analysis and by thin layer chromatography (TLC).

Sf21 cells were infected with the wild type or vG15Bac baculovirus at low multiplicity of infection (2 plaque forming units/cell). Cells were harvested 72 hours post infection (or when the cytopathic effect was total),

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centrifuged and the cell pellet resuspended in 50mM Tris-HCl pH8, followed by 4 cycles of freeze-thaw and then dounce homogenisation. The homogenates were spun down for 10 min at 2000rpm to remove unbroken cells or large aggregates. The supernatant was aliquoted and stored at -70°C and the protein concentration of the homogenates determined by the Pierce bicinchoninic acid (BCA) protein assay (Pierce). CHO, CHOv or CHOg15 cells were grown to confluence and processed as described above. When microsomal fractions were prepared, the supernatants were further centrifuged at 13000rpm for 25 min and the pellet (microsomal fraction) was resuspended in 20mM Hepes, pH7.4.

The colorimetric enzyme assay for measurement of LPAAT activity was performed at room temperature essentially as described in Yamashita et al. (1981) with minor modifications. The enzyme activity can be assayed by measuring the reaction of the thiol group of the released Coenzyme A (CoA) with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) spectrophotometrically giving an increase in absorbance at 413nm. A typical incubation mixture consisted of 100mM Tris-HCl pH7.4, 1mM DTNB, 50µM LPA (oleoyl-sn-glycerol-3-phosphate), 10-45µM acylCoA (Sigma), and 50-150µg cell homogenate, in a total volume of 1ml. DTNB was added as a 0.01M solution in 0.1M potassium phosphate buffer, pH7.0. The reaction was initiated by the addition of acyl-CoA after preincubation of the enzyme with all the other components for 2 min. A molar absorbance of 13,600 M⁻¹ was used to calculate the activity.

To assay the enzyme activity by thin layer chromatography (TLC) the assay procedure was essentially as described above except that 4 µg of enzyme homogenate in a total volume of 10 µl was used, and instead of adding DTNB, 0.02µCi/µl ³H-LPA (1-oleoyl) (New England Nuclear) was used. The assay was also performed using unlabelled LPA and 0.005µCi/µl ¹⁴C-oleoylCoA (Amersham). The reaction was terminated by spotting it onto a silica gel 60 TLC plate

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(Merck) and developed in chloroform:methanol:acetic acid:water (25:10:3:1). To determine the position of substrate and products, LPA and PA standards were loaded, and visualised by exposure to iodine vapours. When ^3H -LPA was used, fluorography was done by immersion of the TLC plate in chloroform containing 10% PPO (2,5-diphenyloxazole) for 10 seconds and then dried without exposure to iodine vapours. To detect the labelled products by autoradiography the TLC plate was exposed to Kodak X-Omat S X-ray film at -70°C for 12-36 hours.

The enzyme activity was linear for at least 3-6 min or 1-2 min when using $45\mu\text{M}$ or $10\mu\text{M}$ acylCoAs, respectively (data not shown). Cell extracts from vG15Bac infected cells, after 6 min of reaction using $10\mu\text{M}$ of acylCoAs, showed that the highest activity was for C16:1 which was 0.532 nmols of CoA released per μg of protein (nmols/ μg protein), and then the rank order was for C16:0 \approx C14:0 \approx C12:0 \approx C18:2>C18:0 \approx C18:3 \approx C20:4 \approx C18:1 (0.389 to 0.285 nmols/ μg protein) with poor or no activity for C20:0 (0.180 nmols/ μg protein) and C24:0 (0.149 nmols/ μg protein), respectively (Fig.6a). The background activity of the wt cell extracts, under these conditions, was from 0.135 (C20:0) to 0.190 (C18:2) nmols/ μg protein. When using $45\mu\text{M}$ acylCoAs after 6 min of reaction the enzyme kinetics showed that hLPAAT had maximal activity for the acyl chains C16:0 \approx C14:0 \approx C16:1>C18:2 \approx C18:3 \approx C12:0 (0.854-0.724 nmols/ μg protein) (Fig.6b), and intermediate activity for C18:1 and C18:0 (0.496 and 0.452 nmols/ μg protein). It showed poor or no activity toward long acyl chains (C20:0, C24:0) (0.262, 0.148 nmols/ μg protein), unless they were unsaturated (C20:4) (0.512 nmols/ μg protein) (Fig.6b). The background activity of the wt cell extracts when using $45\mu\text{M}$ of acylCoAs was the same as that when using $10\mu\text{M}$ of acylCoAs (Fig.6a,b). Similar results were obtained when the reaction was performed using ^3H -LPA and after 6 min the products were detected by TLC (Fig.6c). When the TLC assay

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was performed with unlabelled LPA and ^{14}C -oleoylCoA, specific labelled products (PA) were detected when using vG15Bac cell extracts, which increased as the concentration of LPA was increased (Fig.7). These results confirm previous reported data (Yamashita et al., 1975) obtained using 10-20 μM acylCoAs, where the rank order of specificities of the substrates was: C18:1>C18:2 \approx C16:1>C16:0 \approx C14:0. However, C20:4, C18:0 and C12:0 were described as poor substrates for LPAAT, though the recombinant hLPAAT has significant activity for these substrates. This discrepancy may be due to the previous use of a semipurified preparation of the enzyme from rat liver, that was contaminated with lysophosphatidylcholine acyltransferase (Yamashita et al., 1975). However, in our assay specificity is only due to the recombinant hLPAAT, as the wt baculovirus cell extracts showed no LPAAT activity by TLC (Fig.6c) or showed the same background activity by spectrophotometric analysis when using 10 μM or 45 μM acylCoAs which was independent of the acylCoA used (Fig.6a,b). Glycerophospholipids in animal tissues are known to contain large amounts of arachidonic acid (C20:4) at the *sn*-2 position. It has been proposed that phosphatidylcholine would be deacylated at the *sn*-2 position and then reacylated, by the lysophosphatidylcholine acyltransferase, with C20:4 [for review see (Yamashita et al., 1981)]. Here, we describe that hLPAAT can directly incorporate arachidonoylCoA (C20:4) into phospholipids. Miki et al. (1977), using the semipurified rat liver LPAAT, found that the enzyme had no activity for lysophosphatidylethanolamine or choline. We have confirmed these data for the hLPAAT by spectrophotometric analysis using vG15Bac cell extracts and with 20 μM of C16:1, C12:1, C18:1 and C24:0 as substrates (data not shown).

The activity of the recombinant hLPAAT was also

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assayed in the stable transfected mammalian cell line CHO where it was found that cell extracts from CHO15 showed a higher level of hLPAAT activity (Fig.8) in both the spectrophotometric and TLC assays. The enzyme activity was linear for 6 min of reaction (Fig.8a) and increased in a dose-dependent manner (Fig.8b,c). After 6 min of reaction and when using 45 μ M of oleoylCoA, CHO15 cell extracts showed 1.3 times more activity than CHO or CHO cell extracts, when both 50 or 150 μ g of cell extracts were used (Fig.8c). Similar results were observed when linoleoylCoA was used as substrate (Fig.8b), instead of oleoylCoA (Fig.8a,c), or when a mix of oleoyl and palmitoylLPA was used as substrate instead of oleoylLPA (data not shown). The spectrophotometric assay has also been performed using 100 μ g of microsomal fractions and 4 μ M of linolenoylCoA as substrate. The microsomal fractions obtained from CHO15 cell extracts were found to contain after 5 min of reaction, 1.3 fold more hLPAAT activity compared to microsomal fractions obtained from CHO cell extracts (data not shown). 50 μ g of microsomal fraction prepared from P388G15 and HepG2G15 showed 1.3 and 2.7 fold more hLPAAT activity compared to microsomal fractions obtained from P388 or HepG2 cell lines, respectively.

25 EXAMPLE 6

Cellular localisation

Bursten et al. (1994) have suggested that, in addition to the ER, LPAAT should be located on the plasma membrane as they observed a 1.3-fold activation of LPAAT by IL-1 β when using plasma membrane-enriched compared to crude-microsomal cell fractions. To localise hLPAAT in the cell, immunofluorescence on the different stable CHO cell lines was performed. The immunofluorescence method consisted of

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3 steps. The first involved fixation: cells were washed 3 times in PBS, fixed in 1ml of 4% para-formaldehyde in 250mM Hepes pH7.4 for 30 min at room temperature (RT), then washed again 4 times in PBS, and the cells quenched in 50mM NH₄Cl in PBS for 15 min at RT, followed by 3 washes in PBS. The second step involved incubation with the primary antibody: cells were incubated in 0.2% gelatin, 0.05% saponin in PBS for 15 min at RT to block out non-specific binding. The primary antibodies were centrifuged and diluted in 0.2% gelatin, 0.05% saponin in PBS and cells incubated with the antibodies for 45 min. Cells were rinsed 2 times in PBS containing 0.05% saponin and then washed 3 times in the same solution for 10 min each wash. The third step involved secondary antibody staining: cells were incubated for 15 min in 0.2% gelatin, 0.05% saponin in PBS at RT. The secondary antibody, antimouse IgG conjugated to Fluorescein Isothiocyanate (FITC) isomer I (Sigma), was centrifuged and diluted 1/128 (v/v) in the same solution supplemented with 10% (v/v) goat serum and incubated for 45 min. This step and all the following ones were carried out in the dark. Cells were rinsed 2 times in PBS with 0.05% saponin and washed 2 times for 10 minutes in the same solution. Finally they were washed 2 times for 10 min in PBS. The slides were mounted in Vectashield mounting medium (Vector Lab). The cells were visualised in an Axioscope microscope (Zeiss) or in a Bio Rad 1024 MRC confocal microscope (Sir William Dunn School of Pathology, Oxford).

When cells were not permeabilised the method was essentially the same except that the solutions did not contain detergent (saponin). The first step was staining with the primary antibody at 4°C to avoid permeabilisation and internalisation of the mAbs and membrane proteins. The second step involved the fixation at RT and the third step was the secondary antibody at RT.

In permeabilised cells expressing the tagged hLPAAT, an ER-staining pattern was observed using the T7.TagmAb

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(Fig.9a), that was not present in the control cell lines using the same mAb (data not shown). A similar ER pattern was observed when the three cell lines were stained with the specific ER mAb 1D3 (kindly donated by Dr D. Vaux, Sir William Dunn School of Pathology, Oxford) that labels protein disulphide isomerase (PDI) from the ER (Fig.9b,c,d). The predict-protein program predicts that the T7.Tag should be in the extra-cytoplasmic region. However, immunostaining of unpermeabilised cells or FACS analysis did not show external protein labelling indicating lack of plasma membrane localisation (data not shown). These data suggest that hLPAAT is located in the ER, but possibly very close to the plasma membrane, in which case the enzyme could be activated by direct contact to the plasma membrane itself, or to receptors or receptor complexes located in the plasma membrane. A mechanism has been described for the ryanodine receptor, that when located in the sarcoplasmic reticulum, is activated by direct contact with the dihydropyridine receptor located in the plasma membrane (Coronado et al., 1994). Bursten et al. (1994), when preparing plasma membrane-enriched cell fractions, could have enriched the LPAAT that is very close to the plasma membrane and to the IL-1 β receptors.

The immunofluorescence was also performed on transiently expressed hLPAAT in Cos7 cells (SV40 transformed African Green monkey kidney cells). In order to transiently express hLPAAT Cos7 cells were plated out the night before in DMEM (Sigma) supplemented with 10% (v/v) FCS at 25% confluence, on glass cover slips. The following day cells were washed 3 times with DMEM without FCS prior to the transfection. The medium was poured off and 1ml of DEAE-Dextran transfection mix was added. The transfection mix contained 4 μ l DEAE-Dextran (100mg/ml in PBS), 10 μ l chloroquine (10mM in PBS), 1ml of DMEM (without serum) and 1.5 μ g DNA. The cells were incubated with the transfection mix for 3 hours and then the mix was poured

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off and the cells were washed 3 times with PBS. The PBS was then replaced by 10% (v/v) DMSO in PBS for 2 min and washed again 4 times with PBS. Finally the cells were incubated for 2, 3 and 4 days in DMEM supplemented with 10% (v/v) FCS.

The protein localisation was observed after 2, 3 and 4 days post-transfection (Fig.10). In permeabilised cells an ER staining pattern was observed on each day post-transfection [Fig.10A(2).1, (3).1, (4).1]. These cells, in addition to the ER pattern, showed an intracellular vesicular pattern [Fig.10A(2).2, (3).2, (4).2, (4).3], that in some cells was localised close to the plasma membrane [Fig.10A(4).4]. This could represent a novel mechanism of secretion, directly from the ER to the extracytoplasmic part of the cell and to the intercellular medium, since a lack of Golgi apparatus staining was observed. This possible novel mechanism of secretion will be studied in detail. When the immunolocalisation was performed on non permeabilised cells it was possible to detect externally labelled protein secreted in a "vesicular" form (Fig.10B). These "vesicles" were present in very small numbers after 2 days of transfection [Fig.10B(2).a NP and (2).b NP] and accumulated with time over the next two days [Fig.10B(3).a NP, (3).b NP, (3).c NP and (4).a NP, (4).b NP] After 2 days post-transfection, on non permeabilised cells, only a limited amount of staining close to the cell was observed [Fig.10B(2).b NP]. However, after 3 and 4 days it was possible to observe the cell shape due to the increased accumulation of "vesicular" protein close to the "secretory" cell [Fig.10B(3).a NP, (3).b NP, (3).c NP and (4).a NP, (4).b NP]. It was also possible to detect "ghosts" or stained cell membranes that do not contain a cell [Fig.10B(3).d NP]. The "vesicular" staining was also observed in permeabilised conditions and it was possible to detect this on the surface of the glass slides between cells (see the increased accumulation of background dots

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during the different days in Fig.10A), and very close to the "secretory" cell [Fig.10C(3).b and (4).a]. It was also possible to detect staining on the surface of the cells, which was probably due, not to plasma membrane

5 localisation, but to the secretion of the "vesicles" by the transfected cells followed by their deposition back on top of the cells (note the accumulation of "vesicles" on top of a non permeabilised cell in Fig.10B(4).d NP). Fig.10C shows permeabilised cells after 3 and 4 days post
10 transfection. Fig.10C(3).a shows different "vesicular" staining patterns and Fig.10C(3).b and (4).a show accumulation of "vesicular" secreted protein close to a cell, that in the case of Fig.10C(4).a indicates that the cell is migrating and leaving behind the accumulated
15 protein on the slide. Fig.10C(4).b shows strong staining on a cellular elongation and accumulation of secreted protein close to it. Fig.10C(3).c shows strong staining only close to the plasma membrane and Fig.10C(4).c shows only an accumulation of protein without any cell present.
20 Permeabilised cells transfected with hLPAAT (pcDNA3G15Tag) or pcDNA3 showed a normal ER pattern when stained with the mAb to PDI located in the ER [Fig.10C(3).ER and (V3).ER], and no staining when the cells were not permeabilised (data not shown). These data confirm the topology of the protein
25 shown in Fig.5 and indicate that the C-terminus which contains the T7.Tag, is located in the extracytoplasmic domain. The "secretion" of the protein could be due to

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stimulation or overproduction of the protein that does not occur in the stable transfected CHO cells.

Lysophosphatidic acid-containing vesicles and secretory PLA₂ have been detected in inflammatory fluids of patients with rheumatoid arthritis (Fourcade et al., 1995). These patients could be overexpressing hLPAAT and secreting these vesicles, and this could be a contribution to their inflammatory response, where the PLA₂ has been induced to block the overproduction of PA resulting in the generation of LPA.

EXAMPLE 7

Inhibitory effects of pentoxifylline and lisofylline in LPAAT

Pentoxifylline (PTX) and lisofylline have been described as inhibitors of LPAAT. To measure the inhibitory effects of PTX and lisofylline on hLPAAT activity, DTNB colorimetric assays using the protein expressed in the baculovirus system were performed. PTX was obtained from Sigma and lisofylline was specially synthesised by Dr Parviz Gharagozloo at the MRC Collaborative Centre, Mill Hill, London. The two compounds were dissolved in PBS at 10mM (lisofylline) or 100mM (PTX) as concentrated working solutions.

The colorimetric LPAAT activity assay was performed as

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described before except that 10 μ g of cell extract was used for the PTX assays or 50 μ g of cell extract was used for the lisofylline assays. These were preincubated with different concentrations of lisofylline (0, 1, 10, 100 and 1000 μ M) and PTX (0, 2 and 20 μ M) for 1h at 37°C. After the preincubation time 15 μ M oleoylCoA (PTX assay) or 10 μ M linoleoylCoA (lisofylline assay) was added and the OD at 413nm recorded. The assay was also performed using 50 μ g of cell extract without the preincubation step, adding PTX (0, 1, 10 and 35 μ M) at the same time as the oleoylCoA. The results are shown in Figure 11.

No differences in hLPAAT activity were observed when the vG15BACac, or wild type, baculovirus cell extracts were incubated, during the assay, in the absence or in the presence of up to 35mM PTX (Figure 11a). The same results were observed when the cell extracts were preincubated for one hour, as well as during the assay, with different amounts of PTX (2-20mM) or lisofylline (1-1000 μ M) (Figure 11b and 11c).

Rice et al (1994a), using HPLC analysis of lipids from P388 cells stimulated with LPS, observed an inhibitory effect of PTX and lisofylline on the production of PA. In their studies they used 1mM PTX or 10 μ M lisofylline and they concluded that lisofylline is 800 times more potent than PTX at inhibiting LPAAT activity. In our hands PTX, and lisofylline, cannot inhibit hLPAAT activity when expressed in the baculovirus system, even when used at

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concentrations 20 times (PTX) or 100 times (lisofylline) more than those used by Rice *et al* (1994a). This indicates that these drugs do not inhibit directly the activity of the hLPAAT enzyme.

However, this is not in contradiction to the previously reported results. Rice *et al* (1994a) showed an inhibition of PA produced by the addition of lisofylline after stimulation of P388 cells with LPS, while in a similar analysis Bursten *et al* (1994) showed an inhibition of PA produced by the addition of lisofylline when the same cells were stimulated with IL-1 β or TNF. This indicates that the inhibitory effect of lisofylline (and PTX) could be on the stimulation of LPAAT by these inflammatory agents rather than directly affecting the enzyme itself. This effect could be by inhibition of a protein that activates LPAAT or by inhibition of the interaction of LPAAT with proteins that could activate it. It should be noted that neither of these groups presented data on the effect of the drugs on unstimulated (resting) cells (Rice *et al*, 1994a; Bursten *et al*, 1994).

REFERENCES

- Abraham, E., Bursten, S., Shenkar, R., Allbee, J., Tuder, R., Woodson, P., Guidot, D. M., Rice, G., Singer, J. W., and Repine, J. E. (1995). Phosphatidic acid signaling mediates lung cytokine expression and lung inflammatory injury after hemorrhage in mice. *J Exp Med* 181: 569-575.
- Alberts, B., et al (1989). Molecular Biology of the Cell, 2nd edition.
- Alcami, A., and Smith, G. L. (1992). A soluble receptor for interleukin-1 beta encoded by vaccinia virus: a novel mechanism of virus modulation of the host response to infection. *Cell* 71: 153-167.
- Bursten, S., Weeks, R., West, J., Le, T., Wilson, T., Porubek, D., Bianco, J. A., Singer, J. W., and Rice, G. C. (1994). Potential role for phosphatidic acid in mediating the inflammatory responses to TNF alpha and IL-1 beta. *Circ Shock* 44: 14-29.
- Bursten, S. L., Harris, W. E., Bomsztyk, K., and Lovett, D. (1991). Interleukin-1 rapidly stimulates lysophosphatidate acyltransferase and phosphatidate phosphohydrolase activities in human mesangial cells. *J Biol Chem* 266: 20732-20743.
- Bursten, S. L., Harris, W. E., Resch, K., and Lovett, D. H. (1992). Lipid A activation of glomerular mesangial cells: mimicry of the bioactive lipid, phosphatidic acid. *Am J Physiol* 262: C328-C338.

- 56 -

Campbell, R. D., and Trowsdale, J. (1993) Map of the human MHC. *Immunology Today* , 14: 349-352

Coleman, J. (1992). Characterization of the Escherichia coli gene for 1-acyl-sn-glycerol-3-phosphate acyltransferase (plsC). *Mol Gen Genet* 232: 295-303.

Coronado, R., Morrisette, J., Sukhareva, M., and Vaughan, D. M. (1994). Structure and function of ryanodine receptors. *Am J Physiol* 266: C1485-C1504.

Divecha, N., and Irvine, R. F. (1995). Phospholipid signaling. *Cell* 80: 269-278.

English, D., Cui, Y., and Siddiqui, R. A. (1996). Messenger functions of phosphatidic acid. *Chemistry and Physics of Lipids* 80: 117-132.

Farese, R. V., Konda, T. S., Davis, J. S., Standaert, M. L., Pollet, R. J., and Cooper, D. R. (1987). Insulin rapidly increases diacylglycerol by activating de novo phosphatidic acid synthesis. *Science* 236: 586-589.

Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J. F., Dougherty, B. A., Merrick, J. M., et al. (1995). Whole-genome random sequencing and assembly of Haemophilus influenzae Rd [see comments]. *Science* 269: 496-512.

Fourcade, O., Simon, M. F., Viode, C., Rugani, N., Leballe, F., Ragab, A., Fournie, B., Sarda, L., and Chap, H. (1995). Secretory phospholipase A2 generates the novel lipid

- 57 -

mediator lysophosphatidic acid in membrane microvesicles shed from activated cells. *Cell* 80: 919-927.

Geysen et al, (1986) *Mol. Immunol.* 23: 709-715

Jalink, K., Hordijk, P. L., and Moolenaar, W. H. (1994). Growth factor-like effects of lysophosphatidic acid, a novel lipid mediator. *Biochim Biophys Acta* 1198: 185-196.

Kendall, E., Sargent, C. A., and Campbell, R. D. (1990). Human major histocompatibility complex contains a new cluster of genes between the HLA-D and complement C4 loci. *Nucleic Acids Res* 18: 7251-7257.

Knutzon, D. S., Lardizabal, K. D., Nelsen, J. S., Bleibaum, J. L., Davies, H. M., and Metz, J. G. (1995). Cloning of a coconut endosperm cDNA encoding a 1-acyl-sn-glycerol-3-phosphate acyltransferase that accepts medium-chain-length substrates. *Plant Physiol* 109: 999-1006.

Lassner, M. W., Levering, C. K., Davies, H. M., and Knutzon, D. S. (1995). Lysophosphatidic acid acyltransferase from meadowfoam mediates insertion of erucic acid at the sn-2 position of triacylglycerol in transgenic rapeseed oil. *Plant Physiol* 109: 1389-1394.

Livingstone, C., and Jones, I. (1989). Baculovirus expression vectors with single strand capability. *Nucleic Acids Res* 17: 2366.

Luttinger, A. L., Springer, A. L., and Schmid, M. B. (1991). A cluster of genes that affects nucleoid segregation in *Salmonella typhimurium*. *New Biol* 3: 687-

697.

Miki, Y., Hosaka, K., Yamashita, S., Handa, H., and Numa, S. (1977). Acyl-acceptor specificities of 1-acylglycerolphosphate acyltransferase and 1-acylglycerophosphorylcholine acyltransferase resolved from rat liver microsomes. *Eur J Biochem* 81: 433-441.

Moolenaar, W. H. (1995). Lysophosphatidic acid, a multifunctional phospholipid messenger. *J Biol Chem* 270: 12949-12952.

Nagiec, M. M., Wells, G. B., Lester, R. L., and Dickson, R. C. (1993). A suppressor gene that enables *Saccharomyces cerevisiae* to grow without making sphingolipids encodes a protein that resembles an *Escherichia coli* fatty acyltransferase. *J Biol Chem* 268: 22156-22163.

Pearson, W. R., and Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proc Natl Acad Sci U S A* 85: 2444-2448.

Rice, G. C., Brown, P. A., Nelson, R. J., Bianco, J. A., Singer, J. W., and Bursten, S. (1994a). Protection from endotoxic shock in mice by pharmacologic inhibition of phosphatidic acid. *Proc Natl Acad Sci U S A* 91: 3857-3861.

Rice, G. C., Rosen, J., Weeks, R., Michnick, J., Bursten, S., Bianco, J. A., and Singer, J. W. (1994b). CT-1501R selectively inhibits induced inflammatory monokines in human whole blood ex vivo. *Shock* 1: 254-266.

- 59 -

Rost, B., Casadio, R., Fariselli, P., and Sander, C.

(1995). Transmembrane helices predicted at 95% accuracy. *Protein Sci* 4: 521-533.

Sambrook et al (1989). *Molecular Cloning: A Laboratory Manual*

Singer, J. W., Bursten, S. L., Rice, G. C., Gordon, W. P., and Bianco, J. A. (1994). Inhibitors of intracellular phosphatidic acid production: Novel therapeutics with broad clinical applications. *Expert Opinion on Investigational Drugs* 3: 631-643.

Staden, R. (1987) Computer handling of DNA sequencing projects. In: *Nucleic Acid and Protein Sequence Analysis, a Practical Approach*. IRL Press, Oxford. 173-217.

Swartley, J. S., Balthazar, J. T., Coleman, J., Shafer, W. M., and Stephens, D. S. (1995). Membrane glycerophospholipid biosynthesis in *Neisseria meningitidis* and *Neisseria gonorrhoeae*: Identification, characterization, and metagenesis of a lysophosphatidic acid acyltransferase. *Molecular Microbiology* 18: 401-412.

Tou, J., Jeter, J., Jr., Dola, C. P., and Venkatesh, S. (1991). Accumulation of phosphatidic acid mass and increased de novo synthesis of glycerolipids in platelet-activating-factor-activated human neutrophils. *Biochem J* 280: 625-629.

Yamashita, S., Hosaka, K., Miki, Y., and Numa, S. (1981). Glycerolipid acyltransferases from rat liver: 1-acylglycerophosphate acyltransferase, 1-

- 60 -

acylglycerophosphorylcholine acyltransferase, and diacylglycerol acyltransferase. *Methods Enzymol* 71: 528-536.

Yamashita, S., Nakaya, N., Miki, Y., and Numa, S. (1975). Separation of 1-acylglycerolphosphate acyltransferase and 1-acylglycerolphosphorylcholine acyltransferase of rat liver microsomes. *Proc Natl Acad Sci U S A* 72: 600-603.

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SEQUENCE LISTING

(1) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2045 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 110..958

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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                                     Met Asp
                                     1
TTG TGG CCA GGG GCA TGG ATG CTG CTG CTG CTG CTC TTC CTG CTG CTG      163
Leu Trp Pro Gly Ala Trp Met Leu Leu Leu Leu Leu Phe Leu Leu Leu
      5              10              15
CTC TTC CTG CTG CCC ACC CTG TGG TTC TGC AGC CCC AGT GCC AAG TAC      211
Leu Phe Leu Leu Pro Thr Leu Trp Phe Cys Ser Pro Ser Ala Lys Tyr
      20              25              30
TTC TTC AAG ATG GCC TTC TAC AAT GGC TGG ATC CTC TTC CTG GCT GTG      259
Phe Phe Lys Met Ala Phe Tyr Asn Gly Trp Ile Leu Phe Leu Ala Val
      35              40              45              50
CTC GCC ATC CCT GTG TGT GCC GTG CGA GGA CGC AAC GTC GAG AAC ATG      307
Leu Ala Ile Pro Val Cys Ala Val Arg Gly Arg Asn Val Glu Asn Met
      55              60              65
AAG ATC TTG CGT CTA ATG CTG CTC CAC ATC AAA TAC CTG TAC GGG ATC      355
Lys Ile Leu Arg Leu Met Leu Leu His Ile Lys Tyr Leu Tyr Gly Ile
      70              75              80
CGA GTG GAG GTG CGA GGG GCT CAC CAC TTC CCT CCC TCG CAG CCC TAT      403
Arg Val Glu Val Arg Gly Ala His His Phe Pro Pro Ser Gln Pro Tyr
      85              90              95
GTT GTT GTC TCC AAC CAC CAG AGC TCT CTC GAT CTG CTT GGG ATG ATG      451

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Val Val Val Ser Asn His Gln Ser Ser Leu Asp Leu Leu Gly Met Met	
100 105 110	
GAG GTA CTG CCA GGC CGC TGT GTG CCC ATT GCC AAG CGC GAG CTA CTG	499
Glu Val Leu Pro Gly Arg Cys Val Pro Ile Ala Lys Arg Glu Leu Leu	
115 120 125 130	
TGG GCT GGC TCT GCC GGG CTG GCC TGC TGG CTG GCA GGA GTC ATC TTC	547
Trp Ala Gly Ser Ala Gly Leu Ala Cys Trp Leu Ala Gly Val Ile Phe	
135 140 145	
ATC GAC CGG AAG CGC ACG GGG GAT GCC ATC AGT GTC ATG TCT GAG GTC	595
Ile Asp Arg Lys Arg Thr Gly Asp Ala Ile Ser Val Met Ser Glu Val	
150 155 160	
GCC CAG ACC CTG CTC ACC CAG GAC GTG AGG GTC TGG GTG TTT CCT GAG	643
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 283 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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 20 25 30
 Lys Tyr Phe Phe Lys Met Ala Phe Tyr Asn Gly Trp Ile Leu Phe Leu
 35 40 45
 Ala Val Leu Ala Ile Pro Val Cys Ala Val Arg Gly Arg Asn Val Glu
 50 55 60

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Asn Met Lys Ile Leu Arg Leu Met Leu Leu His Ile Lys Tyr Leu Tyr
 65 70 75 80

Gly Ile Arg Val Glu Val Arg Gly Ala His His Phe Pro Pro Ser Gln
 85 90 95

Pro Tyr Val Val Val Ser Asn His Gln Ser Ser Leu Asp Leu Leu Gly
 100 105 110

Met Met Glu Val Leu Pro Gly Arg Cys Val Pro Ile Ala Lys Arg Glu
 115 120 125

Leu Leu Trp Ala Gly Ser Ala Gly Leu Ala Cys Trp Leu Ala Gly Val
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Ile Phe Ile Asp Arg Lys Arg Thr Gly Asp Ala Ile Ser Val Met Ser
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Glu Val Ala Gln Thr Leu Leu Thr Gln Asp Val Arg Val Trp Val Phe
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Pro Glu Gly Thr Arg Asn His Asn Gly Ser Met Leu Pro Phe Lys Arg
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Val Met Ser Ser Tyr Gln Asp Phe Tyr Cys Lys Lys Glu Arg Arg Phe
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Thr Ser Gly Gln Cys Gln Val Arg Val Leu Pro Pro Val Pro Thr Glu
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Ser Met Leu Thr Val Phe Arg Glu Ile Ser Thr Asp Gly Arg Gly Gly
 260 265 270

Gly Asp Tyr Leu Lys Lys Pro Gly Gly Gly Gly *
 275 280

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CLAIMS

1. An assay for an inhibitor of inflammation mediated via lysophosphatidic acid acyltransferase (LPAAT), which assay comprises:

(a) bringing human LPAAT expressed from a vector comprising a nucleic acid sequence which encodes said LPAAT and which is operably linked to a promoter heterologous to said sequence into contact with a candidate inhibitor in the presence of a lysophosphatidic acid (lysoPA) substrate and a fatty acid cofactor under conditions where, in the absence of inhibitor, the lysoPA is converted to phosphatidic acid (PA), and

(b) comparing the amount of LPAAT activity in the presence and absence of said candidate inhibitor.

2. An assay according to claim 1 wherein the LPAAT is present in growing or intact cells in which the LPAAT has been expressed or in an extract or microsomal fraction from the said cells.

3. An assay according to claim 2 wherein the cells are cells which have little or no endogenous LPAAT activity for the substrate.

4. An assay according to claim 2 wherein the cells are selected from the group consisting of insect cells infected with recombinant baculovirus and mammalian cells.

5. An assay according to claim 4 wherein the cells are selected from the group consisting of CHO, COS, P388 and HepG2 cells.

6. An assay according to claim 1 wherein the LPAAT is present in the form of membrane vesicles.

7. An assay according to any one of the preceding claims wherein a cytokine is present in step (a).

8. An assay according to any one of the preceding claims wherein an inflammatory agent is present in step (a).

9. An assay according to any one of the preceding

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claims wherein the fatty acid cofactor comprises an arachidonoyl group.

10. An assay according to any one of the preceding claims wherein the LPAAT has the amino acid sequence shown in SEQ ID NO: 2.

11. An assay according to claim 10, wherein the said nucleic acid sequence is the sequence from nucleotide 110 to nucleotide 958 of SEQ ID NO: 1.

12. An assay for an activator of inflammation mediated via LPAAT, which assay comprises:

(a) bringing human LPAAT expressed from a vector comprising a nucleic acid sequence which encodes said LPAAT and which is operably linked to a promoter heterologous to said sequence into contact with a candidate activator in the presence of a lysophosphatidic acid (lysoPA) substrate and a fatty acid cofactor under conditions where, in the absence of activator, the lysoPA is converted to phosphatidic acid (PA), and

(b) comparing the amount of LPAAT activity in the presence and absence of said candidate activator.

13. An assay according to claim 12, wherein the LPAAT is present in growing or intact cells in which the LPAAT has been expressed or in an extract or microsomal fraction from the said cells.

14. An assay according to claim 13 wherein the cells are cells which have little or no endogenous LPAAT activity for the substrate.

15. An assay according to claim 13 wherein the cells are selected from the group consisting of insect cells infected with recombinant baculovirus and mammalian cells.

16. An assay according to claim 15 wherein the cells are selected from the group consisting of CHO, COS, P388 and HepG2 cells.

17. An assay according to claim 12 wherein the LPAAT is present in the form of membrane vesicles.

18. An assay according to any one of claims 12 to 17

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wherein a cytokine is present in step (a).

19. An assay according to any one of claims 12 to 18 wherein an inflammatory agent is present in step (a).

20. An assay according to any one of claims 12 to 19 wherein the fatty acid cofactor comprises an arachidonoyl group.

21. An assay according to any one of claims 12 to 20 wherein the LPAAT, has the amino acid sequence shown in SEQ ID NO: 2.

22. An assay according to claim 21 wherein the said nucleic acid sequence is the sequence from nucleotide 110 to nucleotide 958 of SEQ ID NO: 1.

23. A compound found by means of an assay as defined in any one of claims 1 to 11 to be an inhibitor of inflammation mediated via LPAAT.

24. A compound found by means of an assay as defined in any one of claims 12 to 22 to be an activator of inflammation mediated via LPAAT.

25. An isolated polypeptide having LPAAT activity, which has the amino acid sequence shown in SEQ ID NO: 2 or a sequence substantially homologous thereto, or a fragment of either said sequence.

26. An isolated polynucleotide encoding a polypeptide as defined in claim 25.

27. A polynucleotide according to claim 26 which is a DNA sequence.

28. A polynucleotide according to claim 26 which encodes the amino acid sequence of SEQ ID NO: 2.

29. A polynucleotide according to claim 27 which has the coding sequence of SEQ ID NO: 1.

30. A vector incorporating a polynucleotide as defined in any one of claims 26 to 29.

31. A vector according to claim 30 which is an expression vector.

32. A process for the preparation of a polypeptide having LPAAT activity and the amino acid sequence having

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shown in SEQ ID NO: 2 or a sequence substantially homologous thereto, or a fragment of either said sequence, which process comprises:

(i) cultivating a host cell transformed or transfected with an expression vector according to claim 31 under conditions to provide for expression of the said polypeptide, and

(ii) and recovering the expressed polypeptide.

33. A preparation of purified membrane vesicles which comprise a polypeptide as defined in claim 25.

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Fig.1.

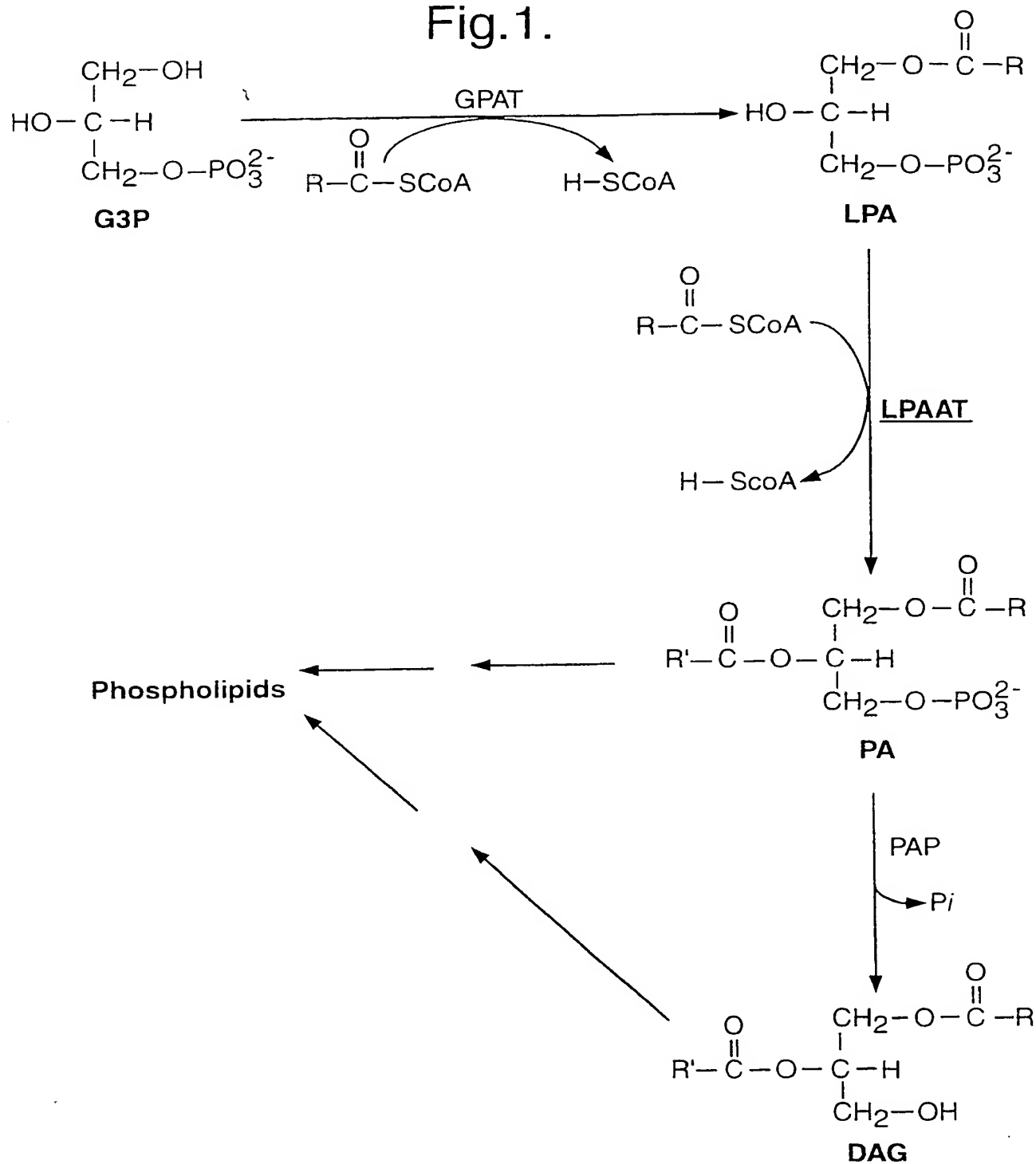


Fig.2a.

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181 CCTGTGGTTCTGCAGCCCCAGTGCCCAAGTACTTCTTCAAGATGGCCTTCTACAATGGCTG I L F L A V L A I P V C A V R G R N V E 64
241 GATCCTCTCTGCTGCTGCTGCCATCCCTGTGTGTCGCTGCCGAGGACGCAACGTCGA N M K I L R L M L L H I K Y L Y G I R V 84
301 GAACATGAAGATCTTGGCTCTAATGCTGCTCCACATCAAAATACCTGTACGGGATCCGAGT E V R G A H F P P S Q P Y V V S N H 104
361 GGAGTGGAGGGGCTACCACTTCCCTCCCTCGCAGCCCTATGTTGTTGTCTCCCAACCA Q S S L D L L G M M E V L P G R C V P I 124
421 CCAGAGCTCTCTCGATCTGCTGGGATGATGGAGTACTGCCAGGCCGCTGTGTGCCCAT A K R E L L W A G S A G L A C W L A G V 144
481 TGCCAGCGGAGCTACTGTGGGTGGCTCTGCCGGCTGGCCTGCTGGCTGGCAGGAGT I F I D R K R T G D A I S V M S E V A Q 164
541 CATCTTCATCGACCGGAAGCGCACGGGGATGCCATCAGTGTGTCATGCTGAGGTGCCCCA T L L T Q D V R V W V F P E G T R N H N 184
601 GACCCGTGCTACCCAGGAGCTGAGGTCTGGGTGTTTCTGAGGGAACGAGAAACCAAA G S M L P F K R G A F H L A V Q A Q V P 204
661 TGGCTCCATGCTGCCCTTCAAACGTGGGGCTTCCATCTTCAGTGCAGGCCCCAGGTTCC I V P I V M S S Y Q D F Y C K K E R R F 224
721 CATTGTCCCCATAGTCATGCTCTCTACCAAGACTTCTACTGCAAGAGGAGCGTCGCTT T S G Q C Q V R V L P P V P T E G L T P 244
781 CACCTCGGGACAATGTCAGGTGGGTGCTGCCCCAGTGCACGGAAGGCTGACACC D D V P A L A D R V R H S M L T V F R E 264
841 AGATGACGTCCCAGCTCTGGCTGACAGAGTCCGGCACTCCATGCTCACTGTTTCCGGGA I S T D G R G G D Y L K K P G G G * 283

Fig.2a (Cont).

901	AATCTCCACTGATGGCCCGGGTGGTGAGCTATCTGAAGAAAGCCCTGGGGCGGTGGGTG
961	AACCCCTGGCTCTGAGCTCTCCCTCCCATCTGTCTCCCATCTTCTCTCCACACACTTACCCACC
11021	CAGTGGGCCCTGAAGCAGGGCCAAACCCTCTTCTCTTGTCTCCCCCTCTCCCCACTTATTCT
11081	CCCTCTTTGGAATCTTCAACTTCTGAAGTGAATGTGGATACAGCGCCACTCCTGCCCCCTC
111141	TTGGCCCCCATCCATGGACTCTTGCCCTCGGTGCAGTTTCCACTCTTGACCCCCACCTCCTTA
11201	CTGTCTTGTCTGTGGGACAGTTGCCCTCCCCCTCATCTCCAGTGACTCAGCCTACACAAGG
11261	GAGGGAAACATTCATCCATCCCCAGTGGAGTCTTTCCTATGTGGTCTTCTCTTACCCCTCTAC
11321	CCCACATTTGGCCAGTGGACTCATCTCATTTCTTTTGGAAACAATCCCCCCCCACTCCAAAAGTCC
11381	ATGGATTCAATGAGCTCATCCCATTTGTGAGGAGGACTTCTCTGCCCTCTCTGGCTTGGAAAGCTG
11441	ATACCTGAAGCACTCCAGGCTCATCTCTGGGAGCTTTTCTCTCAGCACCTTTCACCTTCCCCTC
11501	CCAGTGTAGCCTCTGTCTAGTGGGGCTGGACCCCTTCTTAATTCAAGAGGTCTCATTGCCTGC
11561	CCTTGCCCAGATGCCCAGGGTCTGTGCACTCTCTGGGATACCAGTTTCAGTCTCCACATTTTC
11621	TGGTTTTCTGTCCCCATAGTACAGTTCTTTCAGTGGACATGACCCACCCAGCCCCCTGCA
11681	GCCCTGCTGCACCATCTCACACAGACACAAGGGGAAAGAACAGACATCAGGTGCTGCACCTC
11741	ACTTCTGCCCCCTGGGGAGTTGGGAAAGGAACGAAACCCCTGGCTGGAGGGGATAGGAGGG
11801	CTTTTAAATTATTTCTTTTCTGTGTGAGGCTTCCCCCTCTCTGAGCCAGTTTTCATTTCTCT
11861	TCCTGGTGGCATTAGCCACTCCCTGCTCTCACTCCAGACCTGTTCCTCCACAACCTGGGGAG
11921	GTAGGCTGGGAGCAAAAGGAGAGGGTGGGACCCAGTTTGTGGTGGTTTGTATTATAAT
11981	TATCTGGATAACACAGCAAAAAAACTGAAATAAAAGAGAGAGAGAAAAAATAAAAAA
2041	AAAAA

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Fig.2b.

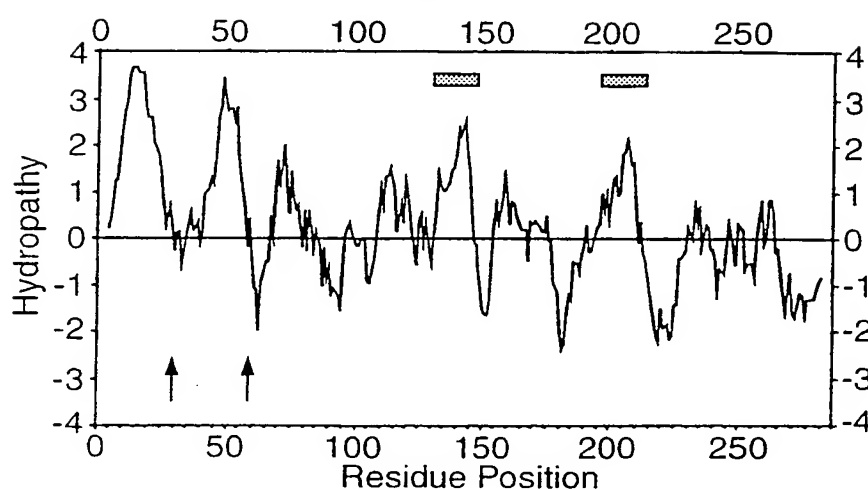


Fig.3a.

$\frac{\%}{\text{SIM}} \backslash \text{ID}$	G15	Scerevisiae	Haemoph	Salmonella	Ecoli	Lalba	Cnucifera	Gonocc	Meningo
G15	—	30.9	30.4	28.1	27.7	28.4	23.6	24.9	24.0
Scerevisiae	54.4	—	29.8	33.1	31.4	28.9	32.8	26.6	23.7
Haemoph	56.7	51.3	—	60.4	61.7	32.1	27.1	21.5	20.2
Salmonella	51.2	56.5	78.8	—	93.9	28.3	28.6	22.1	21.3
Ecoli	51.2	55.2	79.2	96.7	—	28.9	28.8	21.7	20.8
Lalba	53.3	54.5	57.5	51.9	51.9	—	62.0	22.6	22.6
Cnucifera	52.8	55.6	55.1	56.8	54.6	76.0	—	23.1	22.3
Gonocc	47.2	51.7	43.4	44.6	43.8	51.3	48.7	—	97.7
Meningo	47.6	49.8	43.4	44.2	43.3	50.9	47.9	98.8	—

[illegible]

Gonoc	SW	LD	IF	AM	SA	V	Y	P	P	SS	F	-	-	I	A	R	Q	E	I	K	S	W	P	V	L	G	K	M	G	Q	N	A	G	T	V	F	I	N	R	-	R	D	126			
Meningo	SW	LD	IF	AM	SA	V	Y	P	P	SS	F	-	-	I	A	R	Q	E	I	K	S	W	P	V	L	G	K	M	G	Q	N	A	G	T	V	F	I	N	R	-	R	D	126			
Labla	SP	ID	AF	FM	WL	A	P	I	P	SS	F	-	-	G	V	A	K	K	E	I	W	Y	P	L	L	G	Q	L	Y	T	L	A	H	H	I	R	I	D	R	S	N	P	A	A	160	
Cnucifera	SL	VD	IF	EL	I	M	WL	I	P	SS	F	-	-	G	V	A	K	K	E	I	W	Y	P	L	L	G	Q	L	Y	T	L	A	H	H	I	R	I	D	R	S	N	P	A	A	181	
Salmonella	NN	YD	M	V	T	A	S	N	I	V	Q	P	P	T	V	T	V	T	V	T	L	W	I	P	F	F	G	Q	L	Y	W	L	T	G	N	L	L	I	D	R	S	N	P	A	A	124
Ecoli	NN	YD	M	V	T	A	S	N	I	V	Q	P	P	T	V	T	V	T	V	T	L	W	I	P	F	F	G	Q	L	Y	W	L	T	G	N	L	L	I	D	R	S	N	P	A	A	124
Haemoph	NN	YD	M	V	T	A	S	N	I	V	Q	P	P	T	V	T	V	T	V	T	L	W	I	P	F	F	G	Q	L	Y	W	L	T	G	N	L	L	I	D	R	S	N	P	A	A	124
G15	SS	LD	LL	GM	ME	V	L	P	P	SS	F	-	-	I	A	R	Q	E	I	K	S	W	P	V	L	G	K	M	G	Q	N	A	G	T	V	F	I	N	R	-	R	D	155			
Scerevisiae	ST	LD	IF	EL	ML	G	R	I	F	P	SS	F	-	-	I	A	R	Q	E	I	K	S	W	P	V	L	G	K	M	G	Q	N	A	G	T	V	F	I	N	R	-	R	D	133		

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Fig.3c.

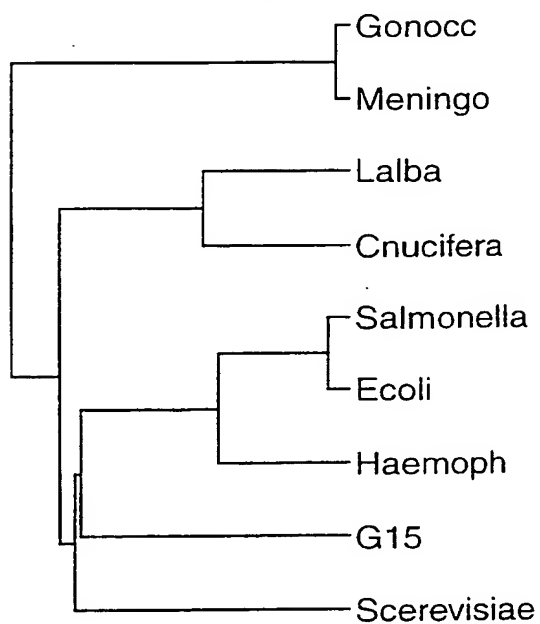


Fig.4a.

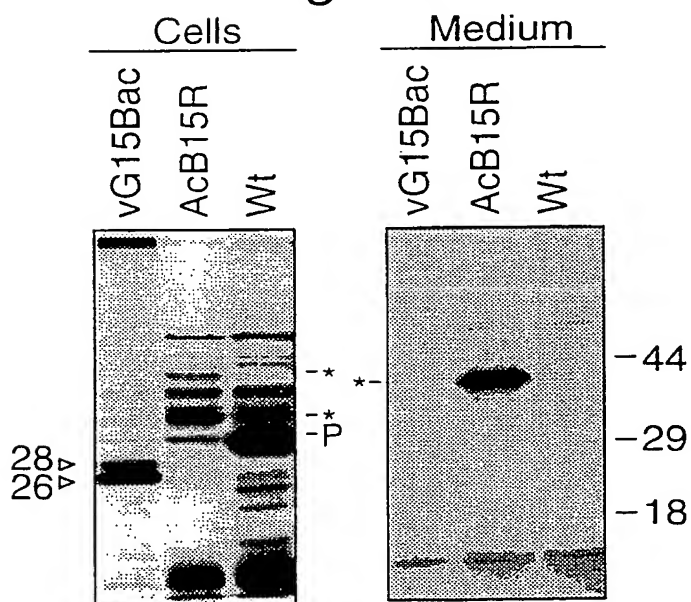
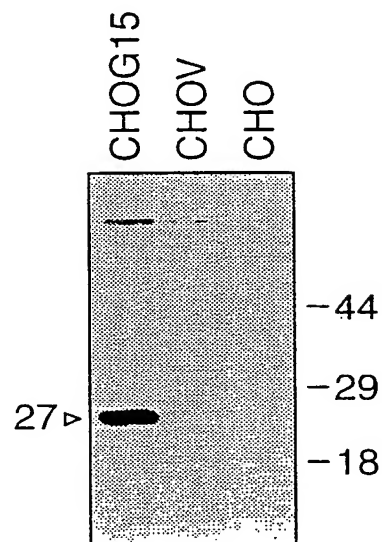


Fig.4b.



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Fig.5.

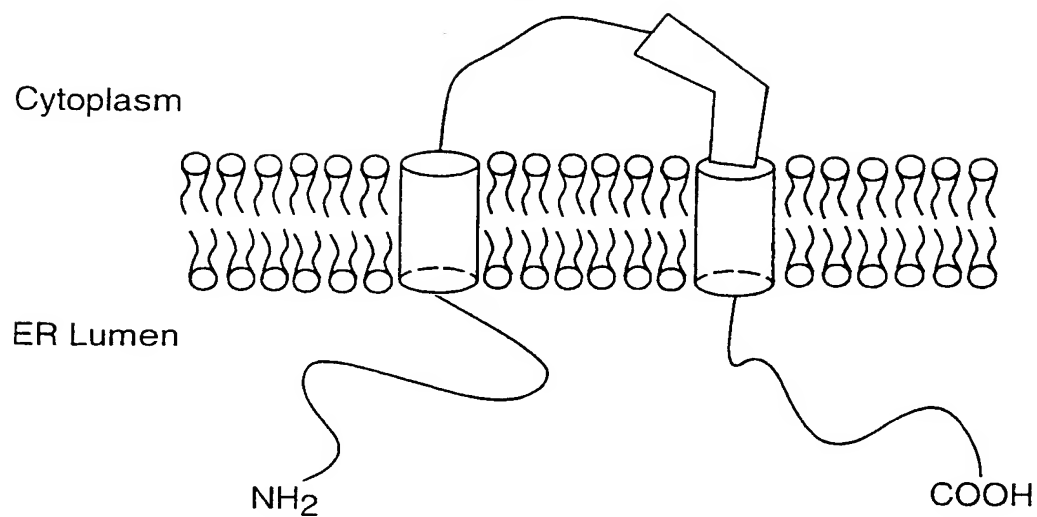
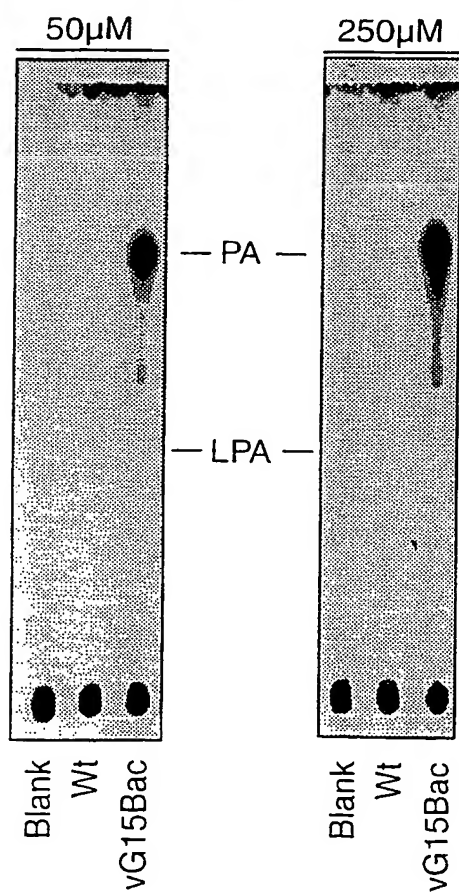
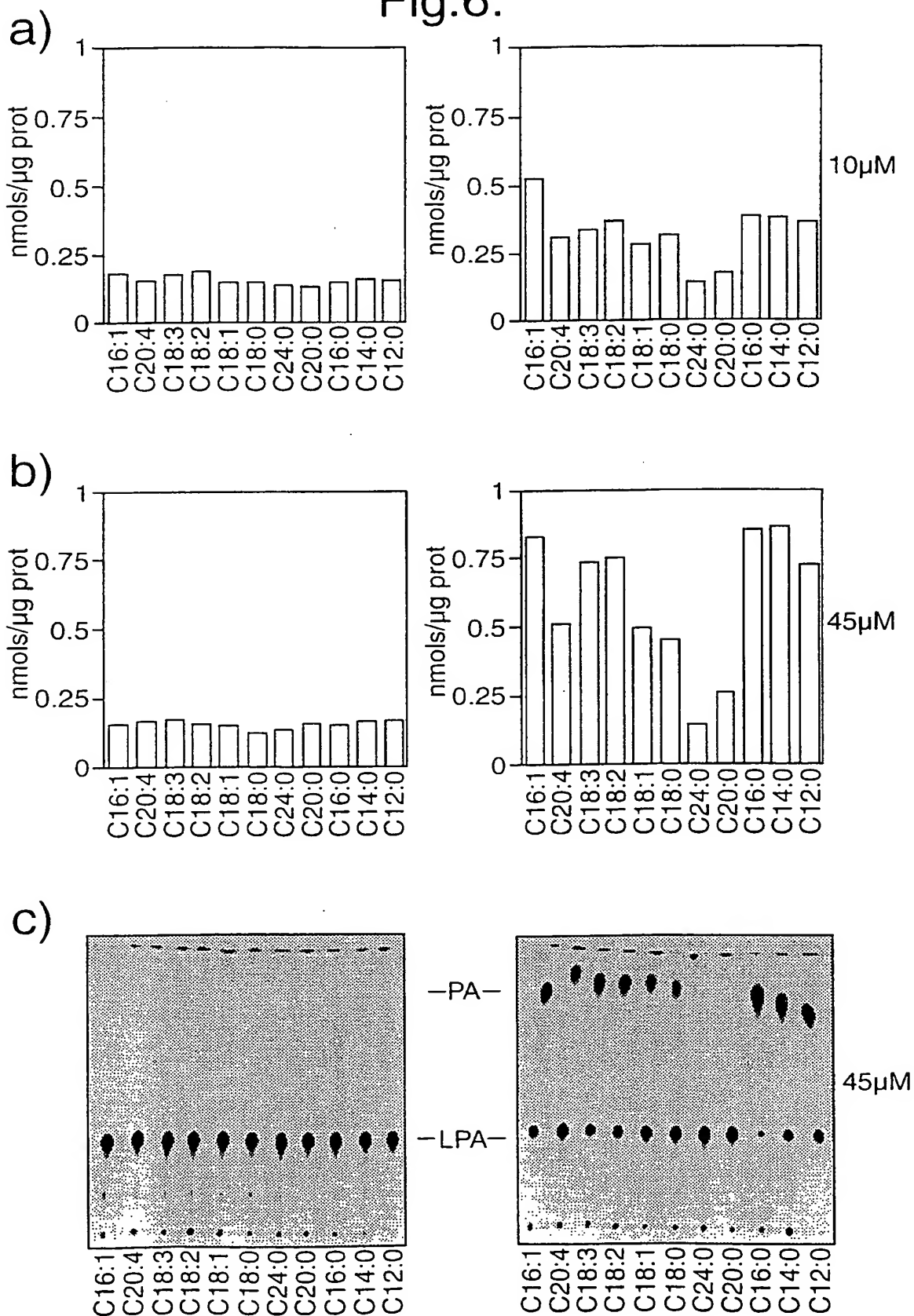


Fig.7.



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Fig.6.



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Fig.8.

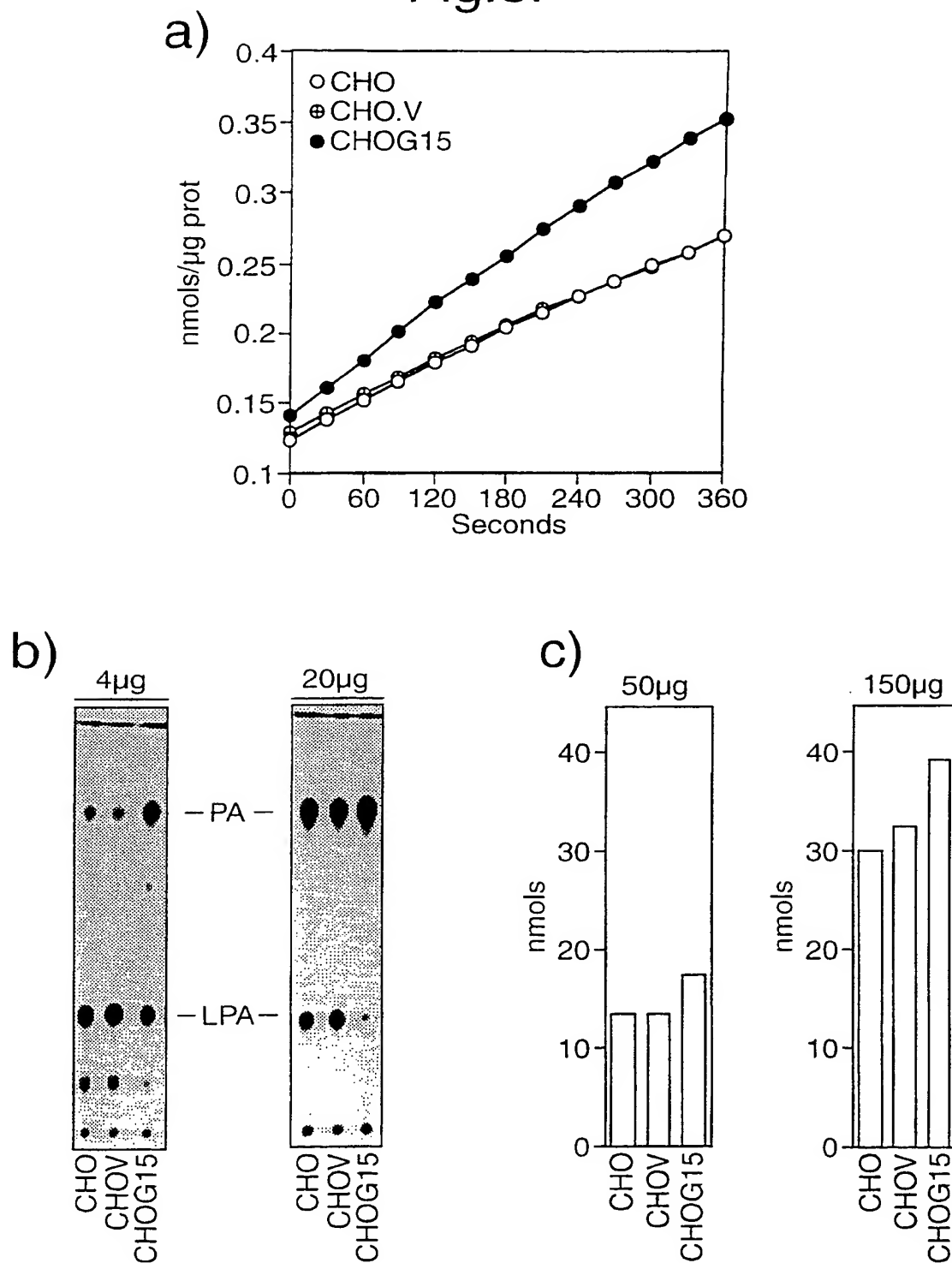
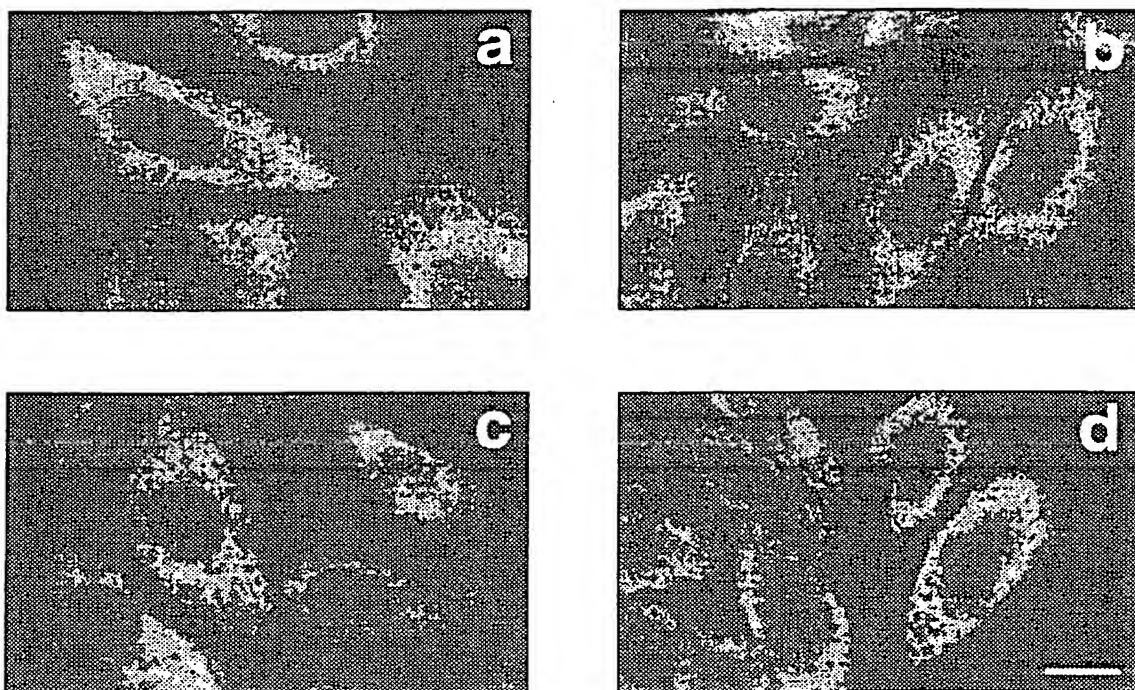
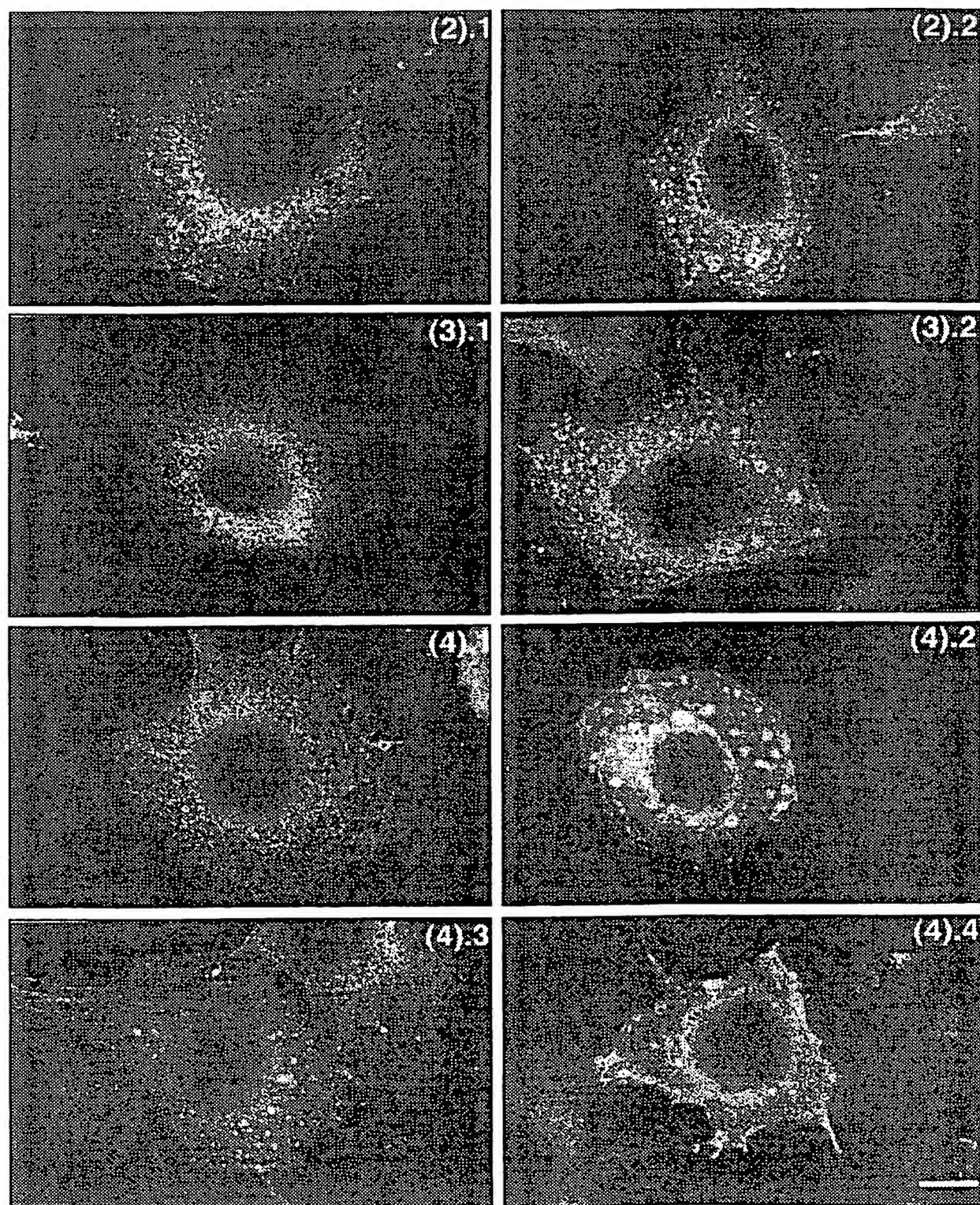


Fig.9.



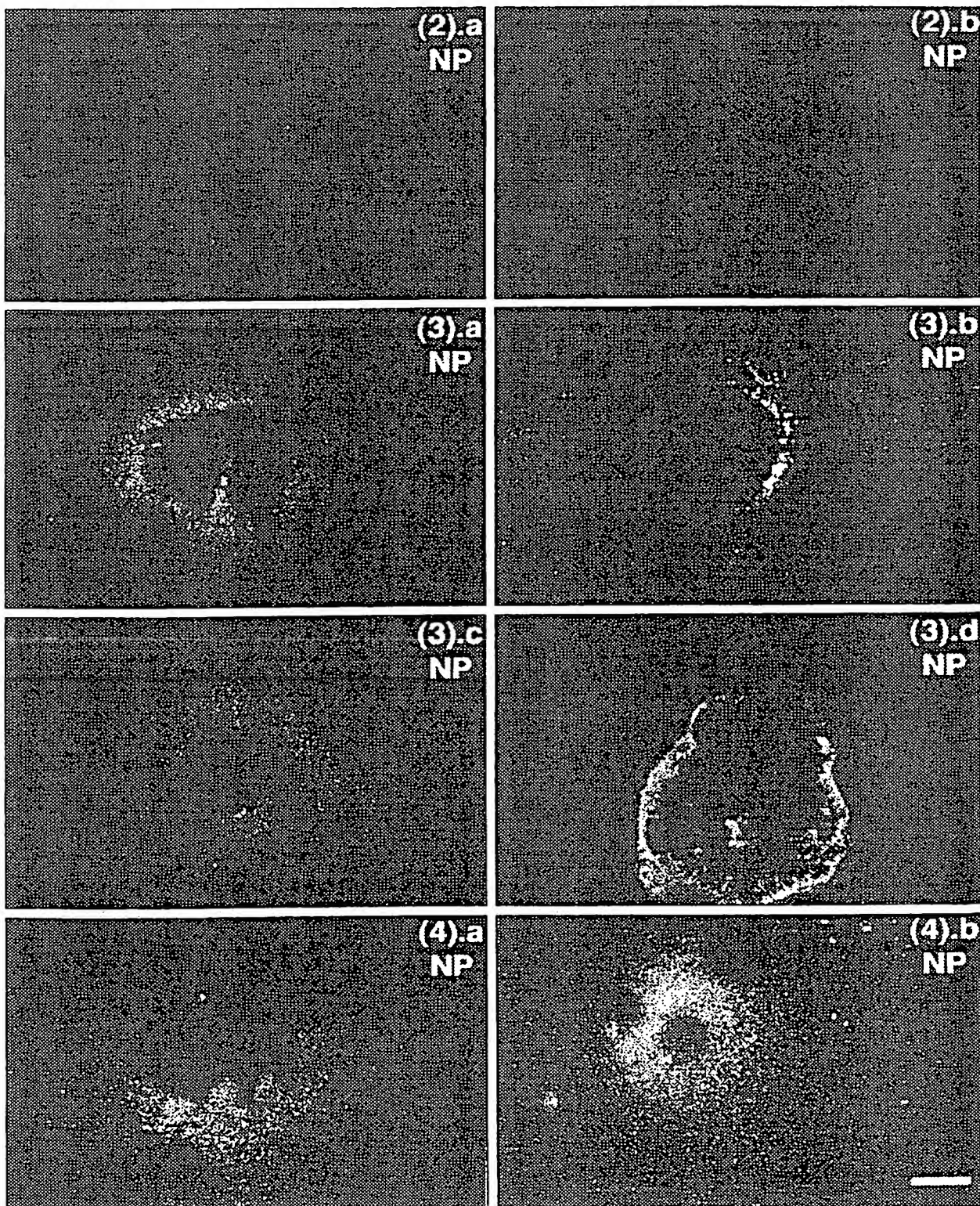
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Fig.10a.



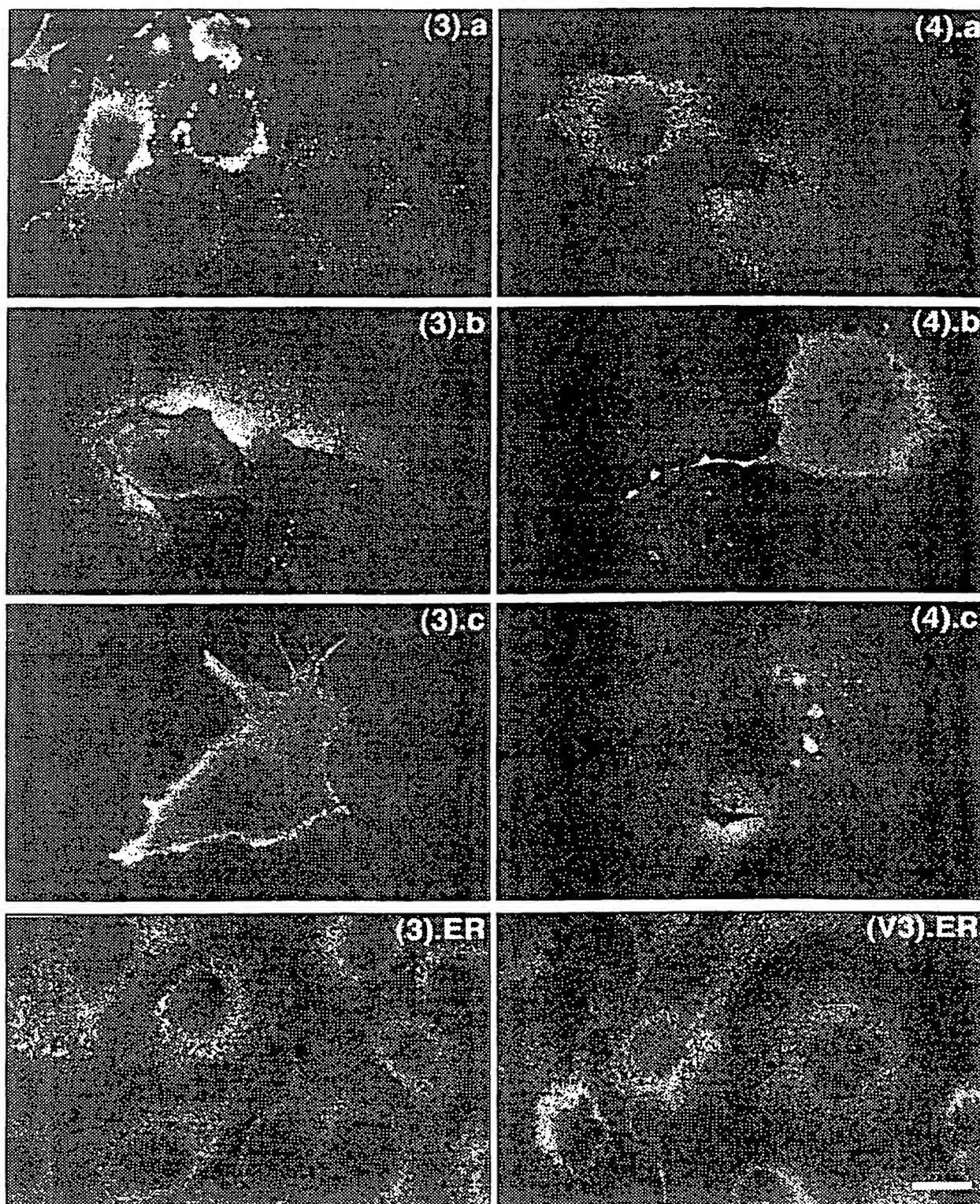
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Fig. 10b.



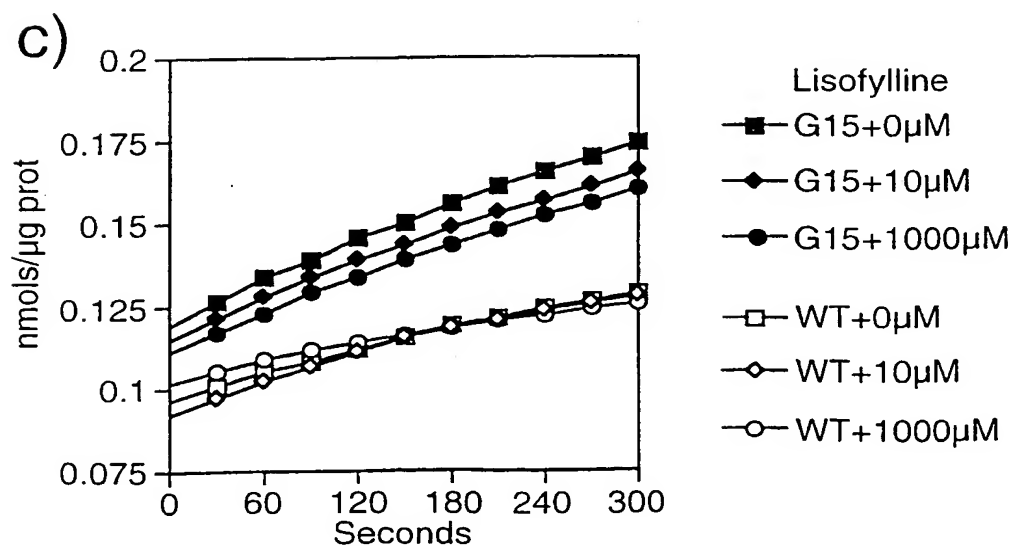
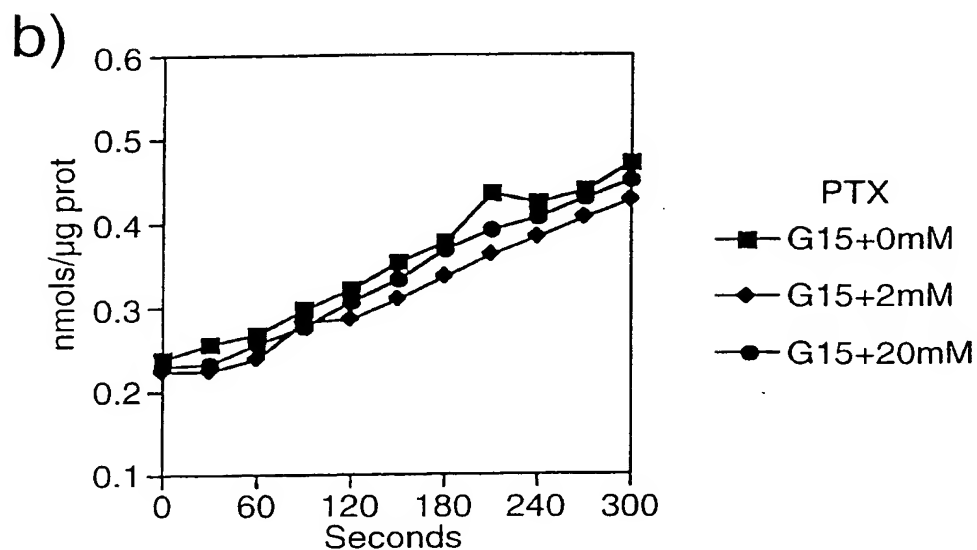
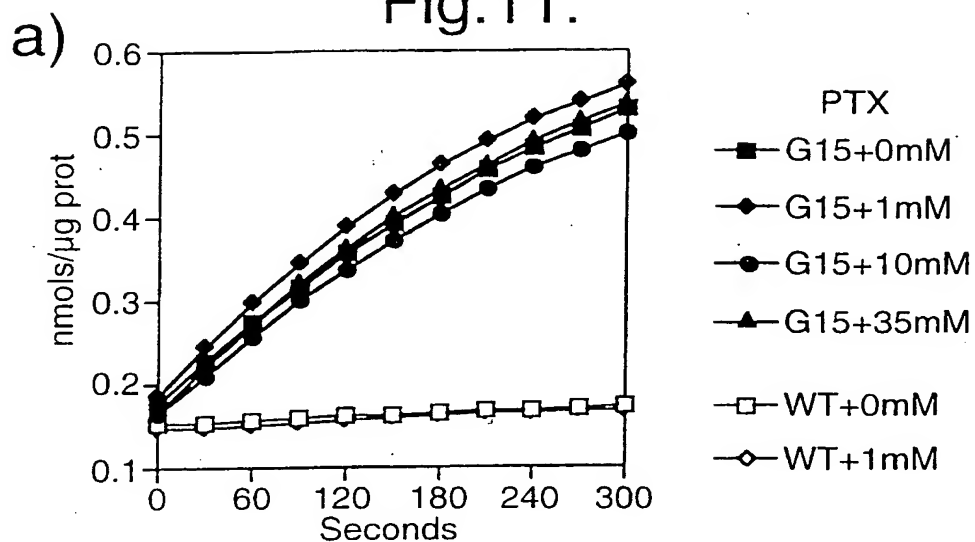
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Fig.10c.



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Fig.11.



INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 97/03471

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/54 C12N9/10 C12Q1/48 C07D473/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12Q A61K C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL Emest6:Hsz78316;access-no:Z78316 NERI C. et al. see Hum.Mol.Genet. 5:1001-1009(1996); (next document) 16-8-1996, last version. XP002030307 see abstract	26,27
X	NERI C. ET ALL: "Survey of CAG/CTG repeats in human cDNAs representing new genes: candidates for inherited neurological diseases." HUMAN MOLECULAR GENETICS, vol. 5, 1996, pages 1001-1009, XP000673216 see the whole document	26,27

-/--

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Date of the actual completion of the international search

7 May 1998

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 97/03471

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL Emest2:Hs08352;access-no:T77083 Hillier L. et al. 'The WasHu-Merck EST Project';25-3-1995 XP002030308 see abstract ---	26,27
X	EP 0 291 873 A (HOECHST AG) 23 November 1988 see the whole document ---	23,24
X	GLENN C RICE ET AL.: "Protection from endotoxic shock in mice by pharmacologic inhibition of phosphatidic acid" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, no. 9, 26 April 1994, WASHINGTON US, pages 3857-3861, XP002064295 cited in the application see the whole document ---	23,24
A	COLEMAN, JACK: "Characterization of the Escherichia coli gene for 1-acyl-sn-glycerol-3-phosphate acyltransferase (plsC)" MOL. GEN. GENET. (1992), 232(2), 295-303 CODEN: MGGEAE;ISSN: 0026-8925, XP002000904 see the whole document ---	1
A	HARES W ET AL: "SUBSTRATE SPECIFICITIES OF THE MEMBRANE-BOUND AND PARTIALLY PURIFIED MICROSOMAL ACYL-COA:1-ACYLGLYCEROL-3-PHOSPHATE ACYLTRANSFERASE FROM ETIOLATED SHOOTS OF PISUM SATIVUM (L.)" PLANTA, vol. 185, 1 January 1991, pages 124-131, XP002000903 see the whole document ---	1
A	WO 95 27791 A (CALGENE INC ;DAVIES HUW MAELOR (US); HAWKINS DEBORAH (US); NELSEN) 19 October 1995 see claims ---	1
A	DE 44 33 307 A (NORDDEUTSCHE PFLANZENZUCHT HAN ;SAATVEREDELUNG LIPPSTADT BREME (DE) 21 March 1996 see claims ---	1
A	WO 95 13075 A (CELL THERAPEUTICS INC) 18 May 1995 cited in the application see claims -----	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 97/03471

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0291873 A	23-11-1988	DE 3717337 A	15-12-1988
		DE 3878980 A	15-04-1993
		DK 277988 A,B,	23-11-1988
		ES 2054731 T	16-08-1994
		IE 62525 B	08-02-1995
		JP 63300965 A	08-12-1988
		PT 87522 B	30-09-1992
		US 4983514 A	08-01-1991
WO 9527791 A	19-10-1995	US 5563058 A	08-10-1996
		CA 2186607 A	19-10-1995
		EP 0754232 A	22-01-1997
		JP 9511650 T	25-11-1997
DE 4433307 A	21-03-1996	AU 3470195 A	09-04-1996
		CA 2200357 A	28-03-1996
		WO 9609394 A	28-03-1996
		EP 0782623 A	09-07-1997
WO 9513075 A	18-05-1995	AU 1090795 A	29-05-1995
		EP 0728003 A	28-08-1996



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(54) Title: LYSOPHOSPHATIDIC ACID ACYLTRANSFERASE GENE AND ITS USE			
(57) Abstract An assay for an inhibitor or activator of inflammation mediated via lysophosphatidic acid acyltransferase (LPAAT) which utilises recombinant human LPAAT. The recombinant human LPAAT is brought into contact with a candidate inhibitor or activator in the presence of a lysophosphatidic acid substrate and a fatty acid cofactor and the amount of LPAAT activity in the presence and absence of the inhibitor or activator is compared. Isolated LPAAT polypeptides, polynucleotides encoding LPAAT and vectors from which the LPAAT is expressed are provided.			

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